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Enhanced Lycopene Production in *Escherichia coli* by Expression of Two MEP Pathway Enzymes from *Vibrio* sp. Dhg

Min Jae Kim [1,†], Myung Hyun Noh [1,†], Sunghwa Woo [1], Hyun Gyu Lim [1,*] and Gyoo Yeol Jung [1,2,*]

1 Department of Chemical Engineering, Pohang University of Science and Technology, 77 Cheongam-Ro, Nam-Gu, Pohang, Gyeongbuk 37673, Korea; mjkim9291@postech.ac.kr (M.J.K.); mh2541@postech.ac.kr (M.H.N.); marswok@postech.ac.kr (S.W.)

2 School of Interdisciplinary Bioscience and Bioengineering, Pohang University of Science and Technology, 77 Cheongam-Ro, Nam-Gu, Pohang, Gyeongbuk 37673, Korea

* Correspondence: hyungyu.lim@postech.ac.kr (H.G.L.); gyjung@postech.ac.kr (G.Y.J.); Tel.: +82-54-279-2391 (G.Y.J.)

† These authors contributed equally to this work.

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Abstract: Microbial production is a promising method that can overcome major limitations in conventional methods of lycopene production, such as low yields and variations in product quality. Significant efforts have been made to improve lycopene production by engineering either the 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway or mevalonate (MVA) pathway in microorganisms. To further improve lycopene production, it is critical to utilize metabolic enzymes with high specific activities. Two enzymes, 1-deoxy-D-xylulose-5-phosphate synthase (Dxs) and farnesyl diphosphate synthase (IspA), are required in lycopene production using MEP pathway. Here, we evaluated the activities of Dxs and IspA of *Vibrio* sp. dhg, a newly isolated and fast-growing microorganism. Considering that the MEP pathway is closely related to the cell membrane and electron transport chain, the activities of the two enzymes of *Vibrio* sp. dhg were expected to be higher than the enzymes of *Escherichia coli*. We found that Dxs and IspA in *Vibrio* sp. dhg exhibited 1.08-fold and 1.38-fold higher catalytic efficiencies, respectively. Consequently, the heterologous overexpression improved the specific lycopene production by 1.88-fold. Our findings could be widely utilized to enhance production of lycopene and other carotenoids.

Keywords: metabolic engineering; lycopene; MEP pathway; 1-deoxy-D-xylulose-5-phosphate synthase; farnesyl diphosphate synthase; *Vibrio* sp. dhg

1. Introduction

Carotenoids are natural pigments present in plants and microorganisms [1]. Some carotenoids are known to function as membrane protective anti-oxidants [2,3], which has led to a high demand for carotenoids for medical and pharmaceutical applications [4,5]. Lycopene is one of the most highly valuable carotenoids owing to its potent antioxidant [6] and disease prevention properties [7,8]. The lycopene market is growing at 3.5 percent annually and is expected to exceed 133 million dollars in 2023, thus supporting high demand and value [9,10]. The industrial production of carotenoids has conventionally been conducted through an extraction from many natural products, such as tomato and watermelon [11]. However, the extraction method does not meet the growing demand due to limited supply of natural products [12,13]. In addition, its stable production is difficult because of fluctuating lycopene content of natural products: 0.00540–1.50 g lycopene/kg of tomato paste [14],

0.03–0.07 g lycopene/kg of watermelon [15]. Chemical synthesis could be considered as an alternative method; however, there are also several critical limitations, including high production cost, low yield, and quality [12,16]. To overcome those limitations, microbial production of lycopene has been studied as a promising strategy because it enables stable production through a simple and sustainable process [17,18].

Lycopene can be biologically synthesized through condensation of two precursors, isopentenyl pyrophosphate (IPP) and dimethylallyl diphosphate (DMAPP), and subsequent chain elongation reactions (Figure 1) [19,20]. The two precursors, IPP and DMAPP, are produced via two distinct pathways [21]. One of the pathways is the mevalonate (MVA) pathway, which is mainly present in eukaryotes and archaea. The MVA pathway utilizes three moles of acetyl-CoA to synthesize one mole of IPP, which is then isomerized to DMAPP by the activity of isopentenyl-diphosphate delta-isomerase (Idi). The other pathway is the 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway, which is mostly present in bacteria and consists of a series of reactions initiated by the condensation of pyruvate and glyceraldehyde-3-phosphate (G-3-P). Although the MEP pathway requires more energy (ATP) and cofactors (NADPH) to synthesize IPP than the MVA pathway [13], the direct use of the two C-3 glycolytic intermediates enables MEP pathway to have a 1.48-fold higher IPP yield (0.83 C-mole/C-mole) than the MVA pathway (0.56 C-mole/C-mole) [13,22]. Since most bacteria showing fast growth inherently possess the MEP pathway for synthesizing their building blocks [21], the MEP pathway has been widely utilized in recent studies for lycopene production [10,23].

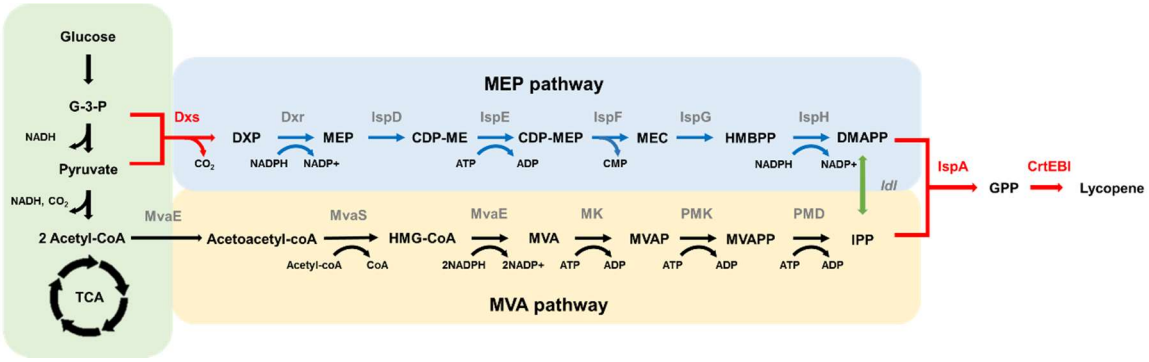

Figure 1. Microbial lycopene production pathway. Lycopene production could be significantly enhanced by heterologous overexpression of CrtEBI and key metabolic enzymes, Dxs and IspA. Dxs, 1-deoxy-D-xylulose-5-phosphate synthase; IspA, farnesyl pyrophosphate synthase; CrtE, geranylgeranyl pyrophosphate synthase; CrtB, phytoene synthase; CrtI, phytoene desaturase.

Significant achievements have been made in lycopene production over the decades by engineering MEP pathway. Heterologous expression of geranylgeranyl diphosphate synthase *(crtE)*, phytoene synthase (*crtB*), and phytoene desaturase (*crtI*) could enable *Escherichia coli* to produce lycopene using inherent MEP pathway [24]. Since then, substantial increase in lycopene production has been reported by overexpression of key metabolic enzymes [7,25]. The condensation of pyruvate and G-3-P has been known as a rate-limiting step of the MEP pathway, and thus overexpression of *dxs gene* (encoding 1-deoxy-D-xylulose-5-phosphate synthase) could enhance lycopene production by to 3.5-fold [25]. Similarly, the catalytic activity of Dxs is known to be subjected to a negative feedback regulation by IPP and DMAPP [26], and expression of *ispA* (encoding farnesyl diphosphate synthase) could improve lycopene production by more than 2-fold [27]. On the other hand, there have been several attempts to increase lycopene production by balancing the expression levels of precursors, pyruvate, and G-3-P. Farmer and Liao were able to significantly improve lycopene production by a dynamic expression control of *pps* gene (encoding phosphoenolpyruvate synthase) [18]; similarly, Jung et al. successfully demonstrated increased lycopene production through a precise expression control of *gapA* (encoding glyceraldehyde-3-phosphate dehydrogenase A) [4].

In addition to those successful approaches, mining and exploiting key metabolic enzymes with high catalytic efficiencies could be an efficient strategy [28,29]. Metabolic flux towards lycopene production could be effectively improved with the use of substitutive key enzymes with higher catalytic activities along with overexpression strategies [7,25,30]. Comprehending the characteristics of the MEP pathway can be of great help in finding enzymes with high catalytic efficiency; carotenoids produced by the MEP pathway are mainly used as essential building blocks for cell membranes and electron transport chain [30]. Considering previous reports, the catalytic efficiencies of essential metabolism enzymes from fast-growing microorganisms are much higher than those from slow-growing microorganisms [31–33]; it can be inferred that fast-growing microorganisms would also possess MEP pathway enzymes with high catalytic efficiency.

Thus, in this study, we have demonstrated the enhancement of lycopene production in *E. coli* by employing key metabolic enzymes from *Vibrio* sp. dhg, which has about 2-fold higher specific growth rate as compared to *E. coli* [23]. Initially, we generated an initial cassette of lycopene production pathway genes, *crtEBI* from *Lamprocystis purpurea* using an inducible promoter and a low-copy plasmid. Thereafter, the key metabolic enzymes, Dxs and IspA were additionally overexpressed in *E. coli* and *Vibrio* sp. dhg, respectively. The expression of those enzymes resulted in a significant increase in lycopene production, especially the expression of *Vibrio* sp. dhg derived enzymes showed a 1.88-fold higher lycopene production. We confirmed that this increase in production could be attributed to higher specific enzyme activity of IspA by subsequent enzyme assays. Our findings can be used not only for enhanced lycopene production, but also for producing numerous other carotenoids.

2. Results

2.1. Enhanced Lycopene Production with Optimization of crtEBI Expression

Previously, a synthetic expression cassette was reported for lycopene production in *E. coli* [4,34]. In this study, the heterologous *crtEBI* from *L. purpurea* was poly-cistronically overexpressed due to metabolic burden (e.g., growth inhibition by lycopene production in early phase) [9,25,35]. Therefore, we optimized lycopene production by modifying the design of expression cassette before comparing the key enzymes. The inducible P_{tac} promoter and a low copy plasmid, pACYCduet-1 [36] were chosen for the stable plasmid maintenance and reduced metabolic burden (p1EBI plasmid for the L1 strain, Table 1) [37]. In addition, to maximize the transcription of *crtE*, *crtB*, and *crtI*, each gene was mono-cistronically expressed under their own P_{tac} promoters and synthetic UTRs (Synthetic UTRs, Table S1; the p2EBI plasmid for the L2 strain, Table 1). The two resulting strains were cultured along with the JYJ0 strain in Luria–Bertani (LB) media for 24 h, and their lycopene production was measured (Figure 2).

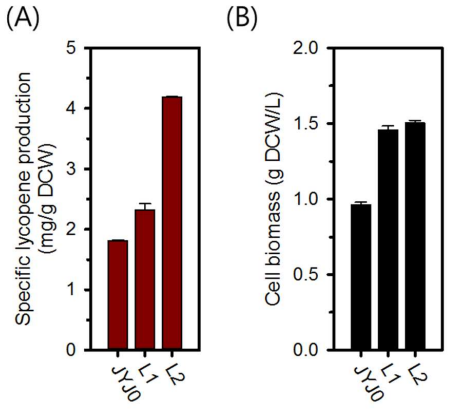

Figure 2. Optimization of *crtEBI* expression. Comparison of specific lycopene production (**A**) and cell biomass (**B**) of the JYJ0, L1, and L2 strains after 24 h cultures. Error bars indicate the standard deviations from three independent cultures.

Table 1. List of strains and plasmids used in this study. (EC: *E. coli*, VDHG: *Vibrio* sp. dhg).

Name	Description	Source
Strains		
E. coli Mach-T1^{R}	Cloning host	Invitrogen
E. coli W3110	Production host, source for *dxs*^{EC} and *ispA*^{EC}	ATCC 9637
Vibrio sp. dhg	Recently isolated fast-growing strain, source for *dxs*^{VDHG} and *ispA*^{VDHG}	[23]
JYJ0	*E. coli* W3110/pCDF_crtEBI	[4]
L1	*E. coli* W3110/p1EBI	This study
L2	*E. coli* W3110/p2EBI	This study
L3	*E. coli* W3110/pdE	This study
L4	*E. coli* W3110/pdV	This study
L5	*E. coli* W3110/piE	This study
L6	*E. coli* W3110/piV	This study
L7	*E. coli* W3110/pdEiE	This study
L8	*E. coli* W3110/pdViV	This study
D1	*E. coli* W3110/pCdEH	This study
D2	*E. coli* W3110/pCdVH	This study
V1	*E. coli* W3110/pCiEH	This study
V2	*E. coli* W3110/pCiVH	This study
P	*E. coli* W3110/pCPFT	This study
Plasmids		
pACYCduet_1	p15A, LacI, Cm^{R}, *E. coli* expression vector	Novagen
p1EBI	p15A, LacI, Cm^{R}, P_{tac}_synUTR$_{crtE}$_*crtE*_ *crtB*_*crtI*_T_{BBa_B1005}	This study
p2EBI	p15A, LacI, Cm^{R}, P_{tac}_synUTR$_{crtE}$_*crtE*_P_{tac}_synUTR$_{crtB}$_*crtB*_ P_{tac}_synUTR$_{crtI}$_*crtI*_T_{BBa_B1005}	This study
pdE	p15A, LacI, Cm^{R}, P_{tac}_synUTR$_{dxsEC}$_*dxs*^{EC} _ P_{tac}_synUTR$_{crtE}$_*crtE*_P_{tac}_synUTR$_{crtB}$_*crtB*_ P_{tac}_synUTR$_{crtI}$_*crtI*_T_{BBa_B1005}	This study
pdV	p15A, LacI, Cm^{R}, P_{tac}_synUTR$_{dxsVDHG}$_*dxs*^{VDHG}_ P_{tac}_synUTR$_{crtE}$_*crtE*_P_{tac}_synUTR$_{crtB}$_*crtB*_ P_{tac}_synUTR$_{crtI}$_*crtI*_T_{BBa_B1005}	This study
piE	p15A, LacI, Cm^{R}, P_{tac}_synUTR$_{ispAEC}$_*ispA*^{EC}_ P_{tac}_synUTR$_{crtE}$_*crtE*_P_{tac}_synUTR$_{crtB}$_*crtB*_P_{tac}_synUTR$_{crtI}$_*crtI*_T_{BBa_B1005}	This study
piV	p15A, LacI, Cm^{R}, P_{tac}_synUTR$_{ispAVDHG}$_*ispA*^{VDHG}_ P_{tac}_synUTR$_{crtE}$_*crtE*_P_{tac}_synUTR$_{crtB}$_*crtB*_ P_{tac}_synUTR$_{crtI}$_*crtI*_T_{BBa_B1005}	This study
pdEiE	p15A, LacI, Cm^{R}, P_{tac}_synUTR$_{ispAEC}$_ *ispA*^{EC}_ P_{tac}_synUTR$_{dxsEC}$_*dxs*^{EC}_ P_{tac}_synUTR$_{crtE}$_*crtE*_P_{tac}_synUTR$_{crtB}$_*crtB*_ P_{tac}_synUTR$_{crtI}$_*crtI*_T_{BBa_B1005}	This study

Table 1. *Cont.*

Name	Description	Source
pdViV	p15A, LacI, Cm^R, P_{tac}_synUTR$_{ispAVDHG}$_ *ispA* ^{VDHG}_ P_{tac}_synUTR$_{dxsVDHG}$_*dxs* ^{VDHG}_ P_{tac}_synUTR$_{crtE}$_*crtE*_P_{tac}_synUTR$_{crtB}$_*crtB*_ P_{tac}_synUTR$_{crtI}$_*crtI*_T_{BBa_B1005}	This study
pCDFduet_1	CloDF13, LacI, Sm^R, *E. coli* expression vector	Novagen
pCDF_crtEBI	CloDF13, LacI, Sm^R, P_{BBa_J23100}_ synUTR$_{crtE}$_*crtE_crtB_crtI*_T_{BBa_B1005}	[4]
pCdEH	CloDF13, LacI, Sm^R, P_{tac}_synUTR$_{dxsEC}$_*dxs*^{EC}_6X His_T_{BBa_B1001}	This study
pCdVH	CloDF13, LacI, Sm^R, P_{tac}_synUTR$_{dxsVDHG}$_*dxs*^{VDHG}_6X His_T_{BBa_B1001}	This study
pCiEH	CloDF13, LacI, Sm^R, P_{tac}_synUTR$_{ispA\,EC}$_*ispA*^{EC}_6X His_T_{BBa_B1001}	This study
pCiVH	CloDF13, LacI, Sm^R, P_{tac}_synUTR$_{ispAVDHG}$_*ispA*^{VDHG}_6X His_T_{BBa_B1001}	This study
pCPFT	CloDF13, LacI, Sm^R, P_{tac}_synUTR$_{PFTase}$_*RAM1*_ T_{BBa_B1001}	This study

The substitution of inducible promoter and plasmid origin led slightly enhanced specific lycopene production in the L1 strain (2.32 mg/g Dry Cell Weight (DCW), 1.28-fold increase) compared to the JYJ0 strain (Figure 2A) [4]. The cell biomass was also increased by 1.52-fold (1.46 g DCW/L, Figure 2B) indicating the reduced metabolic burden. Surprisingly, the monocistronic expression of *crtEBI* could further increase lycopene production in L2 strain (4.18 mg/g DCW), which corresponds to a 2.31-fold increase, while the cell biomass was hardly affected (1.50 g DCW/L). This result implies that the enhanced transcription of *crtB* and *crtI* could improve lycopene production, and thus the L2 strain was utilized for further engineering.

2.2. Validation of Enhanced Lycopene Production with Expression of Dxs and IspA

The key metabolic enzymes for lycopene production, Dxs [25] and IspA [27] were additionally overexpressed in the L2 strain. Considering the characteristics of the MEP pathway [30], it was predicted that those genes from the fast-growing microorganisms could have higher catalytic activities [31,32]. Therefore, we introduced each of the genes derived from *E. coli* W3110 and recently isolated *Vibrio* sp. dhg (Table 1) [23]. The overexpression was conducted by using the P_{tac} promoter and synthetic 5′-UTRs (Table S1) designed for maximum translational levels [38]. The resulting L3–L8 strains (Table 1) were similarly cultivated, and lycopene production was analyzed (Figure 3).

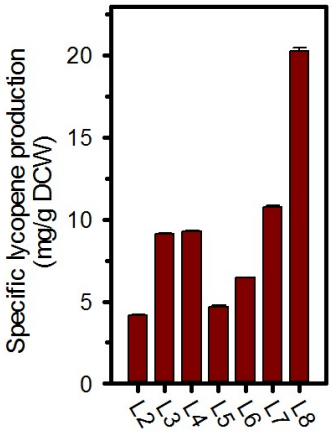

Figure 3. Overexpression of key metabolic enzymes. The specific lycopene production of the L2–L8 strains was plotted from 24-h cultures. Error bars indicate the standard deviations from three independent cultures.

The L3 and L4 strains with overexpression of dxs^{EC} and dxs^{VDHG}, respectively, showed a substantial increase in lycopene production by 2.22-fold as compared to the L2 strain; however, there was no noticeable difference between the two strains (9.12 and 9.26 mg/g DCW, respectively). On the other hand, the expression of *ispA* in the two strains resulted in different lycopene production. The L5 strain with the overexpression of $ispA^{EC}$ showed only a slight increase in lycopene production (4.66 mg/g DCW, 1.11-fold increase), while the L6 strain with $ispA^{VDHG}$ could substantially enhance the production to 6.47 mg/g DCW, which is a 1.55-fold increase.

The co-overexpression of those genes showed a synergetic effect on lycopene production, as previously reported [26]. The L7 strain with co-overexpression in *E. coli* could produce 10.8 mg/g DCW of lycopene, a 2.57-fold increase as compared to the L2 strain, but only a 1.18-fold increase as compared to the L3 strain with dxs^{EC} only expression. In contrast, co-overexpression from *Vibrio* sp. dhg in the L8 strain could significantly enhance lycopene production to 20.3 mg/g DCW. This specific production is not only a noticeable increase as compared to the single dxs^{VDHG} expression (L4 strain) but is also 4.85- and 1.88-fold higher than that obtained from L2 and L6 strains, respectively. Moreover, this production is comparable to the previously reported achievements in LB medium [18,39,40] without the use of other carbon sources or optimization of culture conditions.

2.3. Evaluation of Enzyme Activity

To clarify the enhanced lycopene production with heterologous overexpression of key metabolic enzymes, the enzyme assay was conducted with purified enzymes using 6X His tag (Table 1). The purity and concentration of purified enzymes were validated with sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE, Figure S1) and Bradford assay, respectively. The kinetic properties of k_{cat} and K_m were characterized (Table 2). It should be noted that the assay for Dxs was conducted only for DL-glyceraldehyde because the Dxs is known to have higher affinity for pyruvate [41,42].

As compared to the Dxs^{EC}, Dxs^{VDHG} showed a 1.52-fold higher k_{cat} (3.78 s^{-1}). However, the K_m of Dxs^{VDHG} for DL-glyceraldehyde was found to be 1.40-fold higher (76.50 mM), resulting in a slightly higher catalytic efficiency (k_{cat}/K_m) with a 1.08-fold increase. This observation is consistent with no significant difference in lycopene production between L3 and L4 strains (Figure 3). Considering the high turnover rate (k_{cat}) of Dxs^{VDHG} would be used more effectively with previous precursor balancing strategies [4,18].

Table 2. Kinetic parameters of Dxs and IspA enzymes from *E. coli* and *Vibrio* sp. dhg. Errors indicate the standard deviations from three independent measurements. * DL-glyceraldehyde was alternatively utilized instead of glyceraldehyde-3-phosphate as previously reported [41,43].

Enzyme	Substrate	k_{cat}	K_m	k_{cat}/K_m
Dxs^{EC}	DL-glyceraldehyde *	3.78 (±0.53) s^{-1}	54.48 (±9.42) mM	69.38 (±5.33) μM·s
Dxs^{VDHG}	DL-glyceraldehyde *	5.74 (±0.23) s^{-1}	76.50 (±4.87) mM	75.05 (±3.82) μM·s
$IspA^{EC}$	DMAPP	3.02 (±0.16) s^{-1}	8.79 (±0.42) μM	0.34 (±0.04) μM·s
$IspA^{VDHG}$	DMAPP	4.14 (±0.18) s^{-1}	8.73 (±0.44) μM	0.47 (±0.02) μM·s
$IspA^{EC}$	IPP	3.11 (±0.11) s^{-1}	9.12 (±0.39) μM	0.34 (±0.01) μM·s
$IspA^{VDHG}$	IPP	4.11 (±0.11) s^{-1}	8.45 (±0.43) μM	0.49 (±0.02) μM·s

On the other hand, $IspA^{VDHG}$ showed a 1.35-fold higher k_{cat} (4.13 s^{-1}, an average of all assays) than $IspA^{EC}$ (3.07 s^{-1}). In addition, the K_m values of $IspA^{VDHG}$ for DMAPP (8.73 μM) and IPP (8.45 μM) were slightly lower than $IspA^{EC}$ (8.79 μM and 9.12 μM, respectively). Thus, 1.38- and 1.44-fold higher catalytic efficiencies were characterized in $IspA^{VDHG}$ for each DMAPP and IPP, which support the substantial difference in lycopene production between the L5 and L6 strains (Figure 3). In addition, the large difference between the L7 and L8 strains may reflect differences in catalytic efficiency in addition to the synergetic effects of co-expression [26].

3. Discussion

In this study, we have demonstrated a substantial increase in lycopene production by applying multiple strategies. At first, *crtEBI* was carefully overexpressed by using the low-copy plasmid and the inducible promoter to avoid any potential burden during the initial growth phase. Then, *crtEBI* genes were monocistronically expressed under their own promoters and synthetic 5′-UTR. Thereafter, the heterologous overexpression of *dxs* and *ispA* from the fast-growing *Vibrio* sp. dhg was conducted. The resulting strain showed much higher lycopene production than the starting strain. While the overexpression of *crtEBI* was helpful in increasing lycopene production, the main difference was attributed to the higher catalytic efficiencies of *Vibrio* sp. dhg derived enzymes. These findings can be efficiently applied for the production of other various carotenoids and are not limited to lycopene.

As demonstrated, the utilization of efficient metabolic enzymes has been an elemental strategy in metabolic engineering [7,25,44]. To find high efficiency catalytic enzymes, many studies have been conducted mainly through the directed evolution method based on protein structure [45,46]. In addition, exploiting enzymes from strains with desired characteristics can be a powerful and less labor-intensive strategy [31,32]. In addition to Dxs or IspA, many other efficient enzymes can be excavated from fast-growing microorganism(s). Considering strains with superior properties, such as fast growth [47] and unusual substrate utilization [23] that have been actively isolated in recent years, the similar strategy can be also efficiently utilized for various purposes in metabolic engineering.

4. Materials and Methods

4.1. Reagents and Oligonucleotides

The extraction of plasmid and genomic DNA was conducted with GeneAll^{R} Plasmid SV kit and GeneAll^{R} Exgene^{TM} Cell SV kit (GeneAll, Seoul, Korea), respectively. Q5^{R} High-Fidelity DNA polymerase and NEBuilderR HiFi DNA assembly reagents were purchased from New England Biolabs (Ipswich, MA, USA). Oligonucleotides (Table S2) were synthesized from Cosmogenetech (Seoul, Korea). Other materials were purchased from Sigma-Aldrich (St. Louis, MO, USA).

4.2. Construction of Strains and Plasmids

All bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* Mach-T1^{R} strain (Thermo Scientific, Waltham, MA, USA) was utilized for routine cloning and *E. coli* W3110 strain was

utilized as gene expression and lycopene production host. Genomic DNA of *E. coli* W 3100 and *Vibiro* sp. dhg were purified and utilized as sources of *dxs* and *ispA* (Table S3). Previously constructed JYJ0 strain and pCDF_*crtEBI* plasmid [4] were utilized for lycopene production (Table 1). The RAM1 gene encoding farnesyltransferase (PFTase) was obtained from *Saccharomyces cerevisiae* and codon-optimized for usage in *E. coli* (Table S3). The synthetic promoter (P_{tac}) and terminator (BBa_B1005) were used from the Registry of Standard Biological Parts (http://parts.igem.org). The synthetic 5′-UTRs were designed using the UTR designer for maximum expression (Table S1) [38].

Routine cloning was conducted using NEBuilder^{R} HiFi DNA (New England Biolabs, MA, USA) assembly reagents. To construct the pEBI1 plasmid, the vector fragment was prepared with pACYC_F, pACYC_R, and pACYCduet-1 as a template. *crtE*, *crtB*, and *crtI* fragments were amplified with Tac_crtE_F/R, crtB_F/R, crtI_F/R, respectively. Then, the resulting three fragments and linearized pACYCduet_1 vector were assembled. Similarly, to construct the pEBI2 plasmid, *crtE*, *crtB,* and *crtI* fragments were amplified with Tac_crtE_F/crtE_R, Tac_crtB_F/crtB_R, and Tac_crtI_F/crtI_R, respectively and assembled with vector fragment. For the construction of the pdEiE plasmid, gene fragments were amplified with dxsE_F/R and ispAE_F/R with genomic DNA of *E. coli* W3100 as a template. The resulting fragments were assembled with a vector fragment amplified with crtEBI_F/R and pEBI2 as a template. Likewise, the pdViV plasmid could be constructed with amplified fragments with dxsV_F/R, ispAV_F/R and genomic DNA of *Vibrio* sp. dhg as a template. Plasmids, pdE, and piE were prepared by amplification of pdEiE with dxsE_F/crtEBI_R, and crtEBI_F/ispAE_R, respectively. The pdV and piV plasmids were similarly constructed with dxsV_F/crtEBI_R, crtEBI_F/ispAV_R, and pdViV.

For purification and assay of each enzyme, the 6X His tag was attached at the C-terminus of target protein. A vector fragment prepared with pCDF_HF/R and pCDFduet-1 as a template was assembled with each *E. coli* gene fragment amplified with dxsE_his_F/R and ispAE_his_F/R to construct pCdEH and pCiEH, respectively. Similarly, pCdVH and pCiVH were constructed using *Vibrio* sp. dhg gene fragments amplified with dxsV_his_F/R and ispAV_his_F/R. The pCPFT plasmid also could be constructed with amplified fragment with PFT_his_F/R and synthesized gene fragment as a template.

4.3. Culture Medium and Culture Condition

For lycopene production and protein purification, LB medium (Becton, Dickinson and Company, Bergen County, NJ, USA) containing 10 g/L tryptone, 10 g/L NaCl, and 5 g/L yeast extract was used. To maintain plasmids, appropriate concentrations of antibiotics were added to the medium (50 μg/mL streptomycin and 50 μg/mL chloramphenicol). For seed culture, a single colony was inoculated into a 15-mL test tube containing 3 mL of LB media. After 12 h, the seed culture was inoculated into a 5 mL of fresh LB media to an optical density of 0.05 at 600 nm (OD_{600}). The cells were induced by adding isopropyl-β-ɒ-thiogalactopyranoside (IPTG) at a final concentration of 0.1 mM when OD_{600} reached 0.4. The cultures were grown at 37 °C with agitation at 250 rpm. All experiments were conducted in biological triplicates.

4.4. Quantification of Lycopene Production

For quantification of lycopene production, 1 mL of culture sample was taken and the cell pellet was harvested by centrifugation at 15,814× *g* for 10 min at 4 °C. The cell pellet was washed twice with phosphate buffered saline (PBS). To extract lycopene, cell pellets were suspended in pure 1 mL of pure acetone and incubated at 65 °C heat block for 15 min with intermittent vortexing [4]. The samples were centrifuged at 15,814× *g* for 15 min at room temperature to remove the cell debris. The absorbance of the supernatant was measured at 475 nm UV-1700 spectrophotometer (Shimadzu, Kyoto, Japan). The concentration of lycopene was quantified with a standard curve prepared by using the authentic lycopene. The cell biomass was measured at an absorbance of 600 nm with a spectrophotometer and the concentration of lycopene was normalized to dry cell weight (DCW); one unit of OD_{600} correlates to 0.252 g DCW/L [4].

4.5. Enzyme Assay for Dxs

The D1 and D2 strains were cultivated for 6 h after induction. Cell pellets were harvested by centrifugation at 15,814× *g* for 10 min at 4 °C, and the cell pellet was washed twice with phosphate buffered saline (PBS). The cells were lysed using Bug Buster Master Mix (eBioscience, San Diego, CA, USA) and Dxs enzymes were purified with MagListo™ His-tagged Protein Purification Kit (Bioneer, Daejeon, Korea). Purified enzymes were concentrated using Amicon^{R} ultra centrifugal filters (Merck Millipore, Burlington, MA, USA). SDS-PAGE was conducted to validate the purity of enzymes. The 10% polyacrylamide gel was used for protein separation and PageRuler^{TM} Prestained Protein Ladder (Thermo Scientific) was used as a protein size marker. The protein samples were mixed with the same volume of the SDS gel loading buffer containing 100 mM Tris-HCl (pH 6.8), 4% (*w*/*v*) SDS, 0.2% (*w*/*v*) bromophenol blue, 20% (*v*/*v*) glycerol, and 100 mM dithiothreitol and denatured by incubation at 90 °C for 10 min. The gel was run at 200 V using Mini-PROTEAN^{R} Tetra System (Bio-Rad, Hercules, CA, USA). The concentration of enzymes was measured using Bradford assay (Bio-Rad) and set to the same concentration (0.35 mg/mL).

DXS enzyme assay was performed using reaction mixtures containing 40 mM Tris-HCl (pH 8.0), 2.5 mM $MgCl_2$, 5 mM 2-mercaptoethanol, 1 mM thiamin pyrophosphate, and 100 mM sodium pyruvate DL-glyceraldehyde was added to various concentrations from 0 to 100 mM [41,48]. Then, 10 µL of the purified enzyme was added and final volume was adjusted to 100 µL. The reaction proceeded at 37 °C and the reaction time was varied to 0, 20, 40, and 60 min. After the reaction, the enzymes were inactivated by heating at 95 °C for 10 min. The concentration of pyruvate and DL-glyceraldehyde were measured using the Ultimate 3000 high-performance liquid chromatography (HPLC) system (Dionex, Sunnyvale, CA, USA). As the mobile phase, 5 mM H_2SO_4 was used at a flow rate of 0.6 mL/min; the temperature of the column oven was maintained at 14 °C [49]. The refractive index (RI) was monitored using a Shodex RI-101 detector (Shodex, Klokkerfaldet, Denmark). The experiments were conducted in technical triplicates.

4.6. Enzyme Assay for IspA

Similarly, the V1, V2, and P strains were cultured and purified enzymes were obtained. The concentration of enzymes was set to 0.35 mg/mL. IspA enzyme assay was performed with mixtures containing 125 mM Tris-HCl (pH 8.0), 25 mM $MgCl_2$, 2.5 mM $ZnCl_2$, and 0.2 mM N-dansylated GCVIA [50]. For characterization of K_m for DMAPP, DMAPP was added at various concentrations (~100 µM) with fixed concentration (50 µM) of IPP [51]. Conversely, the concentration of IPP was varied (~100 µM) to validate the K_m for IPP with fixed DMAPP concentration (50 µM). In addition, 20 µL purified PFTase and 10 µL purified IspA were added and the final volume was adjusted to 150 µL. The reaction proceeded at 37 °C and the reaction times varied to 0, 20, 40, and 60 min. After the reaction, the enzymes were inactivated by heating at 95 °C for 10 min. Fluorescence was measured ($\lambda_{excitation}$: 340 nm, $\lambda_{emission}$: 505 nm) using the VICTOR^3 1420 Multilabel Plate Reader (PerkinElmer, Waltham, MA, USA) at room temperature. The activity was calculated using a standard curve prepared with farnesyl pyrophosphate (FPP) instead of DMAPP and IPP [50]. The experiments were conducted in triplicate.

5. Conclusions

Remarkable achievements have been made in microbial lycopene production. In particular, the metabolic key enzymes for production have been characterized and productivity could be significantly enhanced with overexpression strategy. In this study, we newly characterized enzymes with high catalytic efficiency. Considering the characteristics of the MEP pathway, Dxs and IspA from *Vibrio* sp. dhg were heterologously introduced in *E. coli*. The introduction of those enzymes could lead significantly higher lycopene production compared to that of endogenous enzymes. As expected, the higher production was attributed to the higher catalytic efficiencies of *Vibrio* sp. dhg derived enzymes.

Given that two enzymes are not only involved in lycopene synthesis but various carotenoids synthesis, our findings would be effectively applied to carotenoids production.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4344/9/12/1003/s1, Figure S1: SDS-PAGE of total cell lysates and purified enzymes, Table S1: Synthetic 5′-UTR sequences for gene expression, Table S2: Oligonucleotides used in this study, Table S3: The coding sequence of dxs^{VDHG}, $ispA^{VDHG}$, and *RAM1*.

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