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Engineering *Escherichia coli* for selective geraniol production with minimized endogenous dehydrogenation

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ABSTRACT

Geraniol, a monoterpene alcohol, has versatile applications in the fragrance industry, pharmacy and agrochemistry. Moreover, geraniol could be an ideal gasoline alternative. In this study, recombinant overexpression of geranyl diphosphate synthase and the bottom portion of a foreign mevalonate pathway in *Escherichia coli* MG1655 produced 13.3 mg/L of geraniol. Introduction of *Ocimum basilicum* geraniol synthase increased geraniol production to 105.2 mg/L. However, geraniol production encountered a loss from its endogenous dehydrogenization and isomerization into other geranoids (nerol, neral and geranial). Three *E. coli* enzymes (YjgB, YahK and YddN) were identified with high sequence identity to plant geraniol dehydrogenases. YjgB was demonstrated to be the major one responsible for geraniol dehydrogenization. Deletion of yjgB increased geraniol production to 129.7 mg/L. Introduction of the whole mevalonate pathway for enhanced building block synthesis from endogenously synthesized mevalonate improved geraniol production up to 182.5 mg/L in the yjgB mutant after 48 h of culture, which was a double of that obtained in the wild type control (96.5 mg/L). Our strategy for improving geraniol production in engineered *E. coli* should be generalizable for addressing similar problems during metabolic engineering.

1. Introduction

Geraniol (trans-3,7-dimethyl-2,6-octadien-1-ol; $C_{10}H_{18}O$) is an acyclic monoterpene alcohol found in plant essential oils. It is commercially important in flavor and fragrance industries due to its pleasant rose-like odor (Rastogi et al., 2001). Geraniol also exhibits good prospectives in pharmacy and agrochemistry as anticancer drugs (Carnesecchi et al., 2004; Polo et al., 2011), antimicrobial reagents (Togashi et al., 2008; Unlu et al., 2010) and biopesticides (Barnard and Xue, 2004; Papachristos et al., 2004). Besides, geraniol is considered a gasoline alternative superior to ethanol due to its low hygroscopicity, high energy content, and relatively low volatility (Peralta-Yahya and Keasling, 2010).

Geraniol is biosynthesized by geraniol synthase from geranyl diphosphate (GPP) which is the universal precursor of monoterpenes. GPP is synthesized by GPP synthase (GPPS) via head to tail condensation of isopentenyl diphosphate (IPP) with dimethylallyl diphosphate (DMAPP) which can be produced from either the mevalonate (MVA) pathway or the methylerythritol phosphate (MEP) pathway (Goldstein and Brown, 1990; Rohmer, 1999). Although microorganisms do not normally accumulate GPP, mutations in farnesyl diphosphate synthase (FPPS) allow GPP release for monoterpene biosynthesis in recombinant microorganisms harboring monoterpene synthases (Fischer et al., 2011; Oswald et al., 2007; Reiling et al., 2004). GPP accumulation in yeast bearing mutated FPPS enabled geraniol formation in the absence of a heterologous geraniol synthase probably through endogenous dephosphorylation (Blanchard and Karst, 1993; Fischer et al., 2011; Oswald et al., 2007). Similarly, other terpenoid alcohols, such as farnesol and geranylgeraniol, could also be produced in engineered microbes over-expressing FPPS or geranylgeranyl diphosphate synthase (GGPPS) (Muramatsu et al., 2008; Ohto et al., 2009; Tokuhiro et al., 2009; Wang et al., 2010). Thus, it is of interest to see whether geraniol can be produced in the absence of geraniol synthase in a GPP-synthesizing *Escherichia coli* strain. A recent study demonstrated that geraniol could be generated at a level of 0.185 mg/L even in the absence of specific GPPS or mutated FPPS in *E. coli* by simply over-expressing a *Ocimum basilicum* geraniol synthase (ObGES), although the GPP release mechanism remained unclear (Fischer et al., 2013). By co-overexpression of a FPPS mutant and the ObGES in *Saccharomyces cerevisiae*, geraniol production was increased to 5 mg/L after 7 days of culture (Fischer et al., 2011). So

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far, the highest geraniol production has been obtained at a level of 36.04 mg/L in *S. cerevisiae* harboring both FPPS mutant and ObGES after 48 h of culture by overexpressing key rate-limiting enzymes of the mevalonate pathway (Liu et al., 2013). However, this titer is too low to apply to industrial processes. The limited production of geraniol thus prompted us to engineer an effective geraniol biosynthesis pathway in *E. coli*.

Previous studies have individually or partially examined three critical points for increasing geraniol production: increases of building blocks IPP and DMAPP synthesis, GPP synthesis, and GPP conversion to geraniol (Blanchard and Karst, 1993; Fischer et al., 2011, 2013; Liu et al., 2013; Oswald et al., 2007). In this study for maximization of geraniol production, we combined all three aspects and collectively engineered *E. coli* to have an exogenous MVA pathway for increasing building block synthesis, a GPPS mutated from *E. coli* FPPS for GPP synthesis, and a truncated geraniol synthase from *O. basilicum* for efficient conversion of GPP to geraniol (Fig. 1). In addition, we observed endogenous dehydrogenation and isomerization of geraniol in *E. coli*. The geraniol dehydrogenation and isomerization pathway was thus investigated and blocked to further increase geraniol production.

2. Materials and methods

2.1. Bacterial strains and culture conditions

Strains used in this study are listed in Table 1. FRT-flanked kanamycin resistance cassettes amplified from plasmid pKD13 with primer sets yjgB-KO-F/yjgB-KO-R, yddN-KO-F/yddN-KO-R, and yahK-KO-F/yahK-KO-R were used for establishing knock-out strains, MGΔ*yjgB*, MGΔ*yddN* and MGΔ*yahK*, respectively, with a one-step recombinant method (Baba et al., 2006; Datsenko and Wanner, 2000). Kanamycin resistance markers were eliminated from the chromosomes of the knock-out strains according to the reported method (Datsenko and Wanner, 2000). The non-kanamycin resistant mutants were confirmed by PCR using primer sets YjgB-P-F/yjgB-KO-R, YddN-P-F/yddN-KO-R, and YahK-P-F/yahK-KO-R, respectively. Antibiotics with appropriate concentrations (100 mg/L ampicillin and 50 mg/L kanamycin) were added as required in experiments. Glycerol stocks were streaked out on solid LB medium (10 g tryptone, 10 g sodium chloride, 5 g yeast extracts, and 17 g agar per 1 L) and grown overnight. Glass tube (2.5 cm × 15 cm) with plastic cap was used for aerobic culture of the seed and main fermentation. To make seed cultures, individual colonies were picked to inoculate 5 ml 2YT medium (16 g tryptone, 5 g sodium chloride, and 10 g yeast extracts per 1 L) containing 2% glycerol as the main carbon source, and cultured at 30 °C and 250 rpm overnight. Seed from log phase of growth was then inoculated into 5 ml of the same fresh medium to make an initial optical density at 600 nm (OD_{600}) of 0.1 for geranoid feeding experiments, and of 0.4–0.6 optimized for geraniol production. To harvest geranoid from the culture broth during fermentation, two-phase culture was carried out by overlaying 1 ml decane over 5 ml culture broth (Wang et al., 2010). The cultures for geraniol production were performed at 30 °C and 250 rpm for 48 h with initial supplementation of 1 mM IPTG. Mevalonate was prepared from mevalonolactone (Sigma-Aldrich; CAS No. 674-26-0) as described in a previous report (Kim et al., 1992), and initially fed at a concentration of 3.2 mM for culturing of *E. coli* strains harboring the bottom portion of MVA pathway and downstream geraniol synthesis pathway (Fig. 1).

2.2. Plasmids and plasmid construction

E. coli DH5α was used for amplification of all recombinant plasmid constructions. PCR primers and plasmids used in this study are listed in Table 1. *FPPS* (*ispA*) from *E. coli* MG1655 was converted to *GPPS* by site-directed mutation of Ser^{80} into Phe with primers

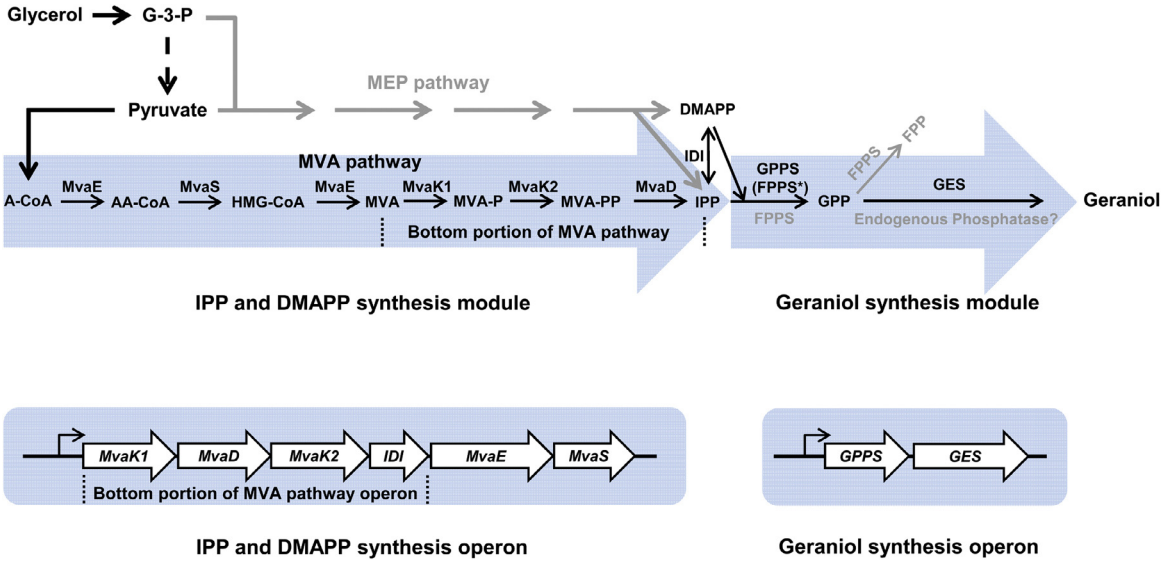

Fig. 1. Modules and corresponding operons of geraniol biosynthesis in engineered *E. coli*. The whole geraniol synthesis pathway is separated into two modules, the upstream IPP/DMAPP synthesis module (left side blue arrow) consisting of an engineered foreign MVA pathway (Yoon et al., 2009) and the downstream geraniol synthesis module (right side blue arrow) consisting of GPP synthase and geraniol synthase. Building blocks of IPP and DMAPP are synthesized from the native MEP pathway (gray arrows) and the foreign MVA pathway using glycerol as the main carbon source. GPP and FPP are synthesized from IPP and DMAPP by GPPS (a mutant derived from FPPS; FPPS*) and FPPS (gray color), respectively. The operons corresponding to the upstream and downstream modules are indicated with solid blue boxes below the pathway diagram. The bottom portion of MVA pathway and its corresponded operon were indicated between two dashed lines, respectively. Open arrows and bent arrows represent genes and promoters, respectively. Abbreviations of the pathway intermediates are as follows: G-3-P, glyceraldehyde-3-phosphate; MEP, methyl-erythritol phosphate; A-CoA, acetyl-CoA; AA-CoA, acetoacetyl-CoA; HMG-CoA, hydroxymethylglutaryl-CoA; MVA, mevalonate; MVA-P, mevalonate 5-phosphate; MVA-PP, mevalonate diphosphate; DMAPP, dimethylallyl diphosphate; IPP, isopentenyl diphosphate; GPP, geranyl diphosphate; FPP, farnesyl diphosphate. Abbreviations of the enzymes are as follows: MvaE, bifunctional acetoacetyl-CoA thiolase and HMG-CoA reductase; MvaS, HMG-CoA synthase; MvaK1, mevalonate kinase; MvaK2, phosphomevalonate kinase; MvaD, mevalonate diphosphate decarboxylase; IDI, IPP isomerase; GPPS, geranyl diphosphate synthase; FPPS, farnesyl diphosphate synthase; GES, geraniol synthase.

Table 1
Primers, plasmids and *E. coli* strains used in this study.

Names	Descriptions	References
Primers[a]		
CR-SacII-F	TTACCCGCGGCCCTCTCACTTCCCTGTT	This study
CR-R	TTTAGCTTCCTTAGCTCCTGA	This study
K28-F	TCAGGAGCTAAGGAAGCTAAAATGAGCCATATTCAACGG	This study
K28-ScaI-R	AAAAGTACTTTAGAAAAACTCATCGAGCATC	This study
ispAS80F-F	GAGTGTATCCACGCTTACTTTTTAATTCATGATGATTTACCG	This study
ispAS80F-R	AAAGTAAGCGTGGATACACTCAACGGCGGCAGC	This study
yddN-KO-F	CATTTGCCTCACCTGCTATGCAGAACATCATCCGAAAAGGAGGAACTATGATTCCGGGGATCCGTCGACC	This study
yddN-KO-R	CCGAACATGGCAGTCGCAGCAAAGGCCTCTTAGTGACGGAAATCAATCACTGTAGGCTGGAGCTGCTTCG	This study
yjgB-KO-F	CTGCCATGCTCTACACTTCCCAAACAACACCAGAGAAGGACCAAAAAATGATTCCGGGGATCCGTCGACC	This study
yjgB-KO-R	GCGCCTCAGATCAGCGCTGCGAATGATTTTCAAAAATCGGCTTTCAACACTGTAGGCTGGAGCTGCTTCG	This study
yahK-KO-F	CATATCAGGCGTTGCCAAATACACATAGCTAATCAGGAGTAAACACAATGATTCCGGGGATCCGTCGACC	This study
GPPS-ERI-F	GGAATTCAGGAGGTAATAAATATGGACTTTCCGCAGC	This study
GPPS-BmI-R	CTGGATCCTTATTTATTACGCTGGATGATG	This study
GES-BmI-F	CTGGATCCAGGAGGTAATAAATATGGAGGAGAGCAGCAGC	This study
GES-XbI-R	GTTTCTAGATTGAGTGAAGAAGAGGGCA	This study
yddN-XbI-F	GCTCTAGAAGGAGGTAATAAATATGAAGGCTGCAGTTGTTAC	This study
yddN-SaI-R	AAGAGTCGACTTAGTGACGGAAATCAATCAC	This study
yjgB-XbI-F	GCTCTAGAAGGAGGTAATAAATATGTCGATGATAAAAAGCTATG	This study
yjgB-SaI-R	AAGAGTCGACTCAAAAATCGGCTTTCAA	This study
yahK-XbI-F	GCTCTAGAAGGAGGTAATAAATATGAAGATCAAAGCTGTTGG	This study
yahK-HndIII-R	TCCGAAGCTTTCAGTCTGTTAGTGTGCGATT	This study
YddN-P-F	CGGAGCTCGCAACAGGCCATTGACGATAA	This study
YjgB-P-F	CGGAGCTCCACTGAAGAGGTATGCGGAAAA	This study
YahK-P-F	CGGAGCTCATTTGAAACGCCTGCAAC	This study
Plasmids		
pSTV28	P_{lac} cloning vector, pACYC184 origin, LacZa, Cm^r	Takara Bio Inc., Otsu, Japan
pSTV28K	*Cat* gene in pSTV28 is replaced by kanamycin antibiotic marker gene from pET28a	This study
TKSN12Didi	pSTV28 K containing *mvaK1*, *mvaK2* and *mvaD* from *S. pneumoniae*, and *idi* from *E. coli*	This study
pSNAK	pTV28 K containing *mvaE* and *mvaS* from *E. faecalis*, *mvaK1*, *mvaK2*, and *mvaD* from *S. pneumoniae*, and *idi* from *E. coli*	This study
pTrc99A	P_{trc} expression vector, pBR322 origin, $lacI^q$, Amp^r	Amann et al. (1988)
pT-yjgB	pTrc99A containing *yjgB* from MG1655	This study
pT-yahK	pTrc99A containing *yahK* from MG1655	This study
pT-yddN	pTrc99A containing *yddN* from MG1655	This study
pT-GPS	pTrc99A containing *GPPS* mutated from *E. coli ispA*	This study
pT-GPSGES	pTrc99A containing *GPPS* and t*ObGES*	This study
Strains		
MG1655	*E. coli* K-12; $F^-\lambda^-$, ilvG$^-$, *rfb*-50, *rph*-1	ATCC 700926[b]
DH5α	*E. coli* K-12; 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	ATCC 98040
DHpTrc	MG1655 harboring pTrc99A	This study
DHyjgB	MG1655 harboring pT-yjgB	This study
DHyddN	MG1655 harboring pT-yddN	This study
DHyahK	MG1655 harboring pT-yahK	This study
GDPB	MG1655 harboring pSTKSN12Didi and pT-GPS	This study
GEOLB	MG1655 harboring pSTKSN12Didi and pT-GPSGES	This study
GEOLW	MG1655 harboring pSNAK and pT-GPSGES	This study
MGΔ*yjgB*	MG1655 Δ*yjgB*	This study
MGΔ*yahK*	MG1655 Δ*yahK*	This study
MGΔ*yddN*	MG1655 Δ*yddN*	This study
KGEOLB	MGΔ*yjgB* harboring pSTKSN12Didi and pT-GPSGES	This study
KGEOLW	MGΔ*yjgB* harboring pSNAK and pT-GPSGES	This study

[a] Restriction sites are underlined.
[b] ATCC, American type culture collection.

ispAS80F-F and ispAS80F-R (Reiling et al., 2004). The geraniol synthase gene (*ObGES*) was kindly provided by Prof. Eran Pichersky (University of Michigan) (Iijima et al., 2004). The N-terminal sequence consisting of 65 amino acids of ObGES was predicted as a chloroplast signal peptide and removed by PCR using primers GES-BmI-F and GES-XbI-R to make a truncated form (tObGES). *GPPS* was amplified with primers GPPS-ERI-F and GPPS-BmI-R, and inserted between *Eco*RI and *Bam*HI restriction sites of pTrc99A vector (Amann et al., 1988) forming the plasmid pT-GPS. Gene *tObGES* was then cloned into pT-GPS and assembled downstream of *GPPS* between *Bam*HI and *Sal*I restriction sites to form the geraniol synthesis plasmid, pT-GPSGES (Fig. 1). Genes involved in geraniol dehydrogenation in *E. coli* (*yjgB*, *yahK* and *yddN*) were cloned into restriction sites of *Xba*I/*Sal*I, *Xba*I/*Hin*dIII, and *Xba*I/*Sal*I of pTrc99A using a pair of primers yjgB-XbI-F/yjgB-SaI-R, yahK-XbI-F/yahK-HndIII-R, and yddN-XbI-F/yddN-SaI-R, respectively.

The MVA pathway plasmids, pSSN12Didi and pSNA, were previously constructed by Yoon et al. (2009, 2006). In plasmid pSSN12Didi, three genes (*mvaK1*, *mvaK2* and *mvaD*) of *Streptococcus pneumonia* encoding the bottom portion of MVA pathway were constructed into vector pSTV28 and controlled under the *lac* promoter (Yoon et al., 2006). Genes (*mvaE* and *mvaS*) of *Enterococcus faecalis* encoding the top portion of MVA pathway were further constructed into pSSN12Didi, resulting in plasmid pSNA (Yoon et al., 2009). The *cat* gene in pSTV28 encoding chloramphenicol acetyltransferase (CAT) is known to be responsible for chloramphenicol antibiotic resistance. However, the CAT was observed to be of nonspecific esterification activity toward some terpene alcohols, such

as esterification of perillyl alcohol into perillyl acetate (Alonso-Gutierrez et al., 2013), and geraniol into geranyl acetate in this study (Supporting information, Fig. S1). The *cat* gene in the MVA pathway plasmids was subsequently replaced with a kanamycin resistance gene as following procedures: (1) a DNA fragment covering the restriction site *Sac*II upstream of the *cat* gene in pSTV28 was amplified with the primer set of CR-SacII-F/CR-R; (2) the kanamycin gene was amplified from plasmid pET28a with the primer set of K28-F/K28-ScaI-R; (3) these two DNA fragments were then overlapped by PCR with the primer set of CR-SacII-F/K28-ScaI-R to form a kanamycin resistance DNA fragment containing the restriction sites, *Sac*II and *Sca*I; (4) the resulting DNA fragment was then inserted between *Sac*II and *Sca*I restriction sites of plasmids pSTV28, pSSN12Didi and pSNA to replace the original *cat* gene. The resulting plasmids were named as pSTV28 K, pSTKSN12Didi and pSNAK, respectively.

2.3. Geranoid feeding experiments

One milliliter of decane containing about 200 mg per liter medium of each geranoid (geraniol, nerol and citral) was fed to the cultures of strains DH5α, MG1655, MGΔ*yjgB*, MGΔ*yddN*, MGΔ*yahK*, DHpTrc, DHyjgB, DHyddN and DHyahK for investigation of the conversion patterns of each fed geranoid. IPTG was added at a final concentration of 0.1 mM for inducing the expression of each geraniol dehydrogenase candidate in strains DHyjgB, DHyddN and DHyahK using DHpTrc as a control. The decane phase was then subjected to gas chromatography (GC) for analysis of compositional variation among the target compound and its derivatives during the culture. The standard compounds were ordered from Sigma–Aldrich, US (geraniol, CAS No. 106-24-1; citral, a mixture of geranial and neral, CAS No. 5392-40-5; nerol, CAS No. 106-25-2).

2.4. Analysis of geraniol and its derivatives

After the two-phase culture for geraniol production, the decane phase was separated and collected by centrifugation at 13,000 rpm for 15 min. After decane separation, the cell pellets in the remaining culture broth were re-suspended by vortex. After the re-suspension, the residual geraniol in the culture broth phase was further extracted by thoroughly mixing ethyl acetate with an equal volume of culture broth, and separated by centrifugation. The decane phase and ethyl acetate extraction were subsequently subjected to GC and GC–mass spectrometry (GC–MS). During the two-phase culture, almost all of the geranoids produced or fed were extracted into the decane phase, only trace or undetectable amount of them remained in cell pellets. Therefore, the production of geraniol in the decane phase was estimated and quantified for all experiments. Geraniol and its derivatives was identified on a Mass Spectrometer GC (GCMS-QP2010, SHIMADZU, Japan), and quantified using Agilent Technologies 7890A Gas Chromatograph equipped with a flame ionization detector (FID). In the GC analysis, two microliters of each sample were separated on a 19091J-413 HP-5 column (length, 30 m; internal diameter, 0.32 mm; film thickness, 250 μm). The oven temperature was initially held at 80 °C for 1 min and sequentially increased at the rate of 10 °C/min to 180 °C and 30 °C/min to 250 °C. Nitrogen was used as the carrier gas with an inlet pressure of 39.0 psi. The detector temperature was maintained at 280 °C. The standard curves of geraniol and nerol were constructed using decane as the solvent for quantitative calculation of their production (Supporting information, Fig. S2). The authentic standards of neral and geranial are commercially unavailable. Since neral and geranial are isomers of each other and have similar GC sensitivities, the standard curve of citral using decane as the solvent was constructed for approximate calculation of the production of neral and geranial (Supporting information, Fig. S2).

3. Results and discussion

3.1. Geraniol biosynthesis in the presence and absence of geraniol synthase from bottom portion of MVA pathway in engineered E. coli

Geraniol is known as a highly toxic compound to *E. coli* (Shah et al., 2013a,b). A decane overlay two-phase culture system, which has been widely used for efficiently harvesting toxic or volatile terpenoids from engineered microorganisms (Alonso-Gutierrez et al., 2013; Wang et al., 2011, 2010), was carried out during geraniol production in *E. coli*. Geraniol formation via endogenous dephosphorylation of GPP has been observed in yeast bearing mutated FPPS (Blanchard and Karst, 1993; Fischer et al., 2011; Oswald et al., 2007). However, geraniol production was not detected during 48 h of culture in *E. coli* overexpressing the GPPS mutated from *E. coli* FPPS (IspA) under the strong *trc* promoter.

The failure of geraniol production might be ascribed to the limited supply of IPP and DMAPP from the native MEP pathway for synthesis of sufficient GPP. A bottom portion of MVA pathway (pSTKSN12Didi) was thus introduced for adequate supply of IPP and DMAPP using fed mevalonate as the substrate (Wang et al., 2010; Yoon et al., 2006). Encouragingly, co-overexpression of the GPPS (pT-GPS) and the bottom MVA pathway (pSTKSN12Didi) in the strain GDPB enabled detectable geraniol formation in the decane layer after 6 h of culture. After 36 h of culture, geraniol production was increased to around 13.3 mg/L and not further increased (Table 2). However, this titer of geraniol production is still very low, which was probably due to the low native catalytic activity for GPP conversion to geraniol in *E. coli*. To enhance the conversion of GPP to geraniol, *tObGES* was introduced into the GPPS expression vector (pT-GPS) to form a geraniol synthesis operon (pT-GPSGES) (Fig. 1). Co-overexpression of pSTKSN12Didi and pT-GPSGES in GEOLB significantly increased geraniol production to a titer of 117 mg/L at 36 h with supplementation of 3.2 mM mevalonate (Table 2). However in the next 12 h, geraniol production was slightly reduced to 105.2 mg/L. It was noted that some other peaks appeared in addition to the geraniol peak during geraniol production in both strain GDPB and GEOLB. It thus raises a question on whether the generation of these new compounds is related to the reduced geraniol production.

3.2. Endogenous geraniol dehydrogenation and geranoid formation in E. coli

Those new compounds were subsequently identified as nerol, neral and geranial by GC and GC–MS analyses (Fig. 2), which together with geraniol are collectively called geranoids. Nerol is the *cis*-isomer of geraniol, while geranial and neral are the oxidative products of geraniol and nerol, respectively. Geranial and neral did not seem to be directly synthesized by tObGES, because these two geranoids were also observed in the culture of GDPB in the absence of tObGES (Table 2). As it can be seen in Table 2, geranial was observed following the formation of geraniol. Therefore, it was suspected that the formation of geranial in *E. coli* was due to the endogenous dehydrogenation of geraniol. To address this hypothesis, authentic geraniol standard was fed to culture of *E. coli* DH5α to observe its dehydrogenation. As expected, the amount of fed geraniol was decreased over culture time along with the accumulation of other geranoids (Table 3), suggesting that geraniol could be dehydrogenated and transformed into other geranoids in *E. coli*. Thus, the production of total geranoids in GDPB and GEOLB at 48 h of culture was quantified as 20.8 mg/L and 134.0 mg/L, respectively (Table 2).

Table 2
Geranoid production and cell growth of *E. coli* strains GDPB and GEOLB.

Time	GPB						GEOLB					
	Nerol (mg/L)	Neral (mg/L)	Geraniol (mg/L)	Geranial (mg/L)	Total geranoids (mg/L)	Cell growth (OD_{600})	Nerol (mg/L)	Neral (mg/L)	Geraniol (mg/L)	Geranial (mg/L)	Total geranoids (mg/L)	Cell growth (OD_{600})
6 h	ND[a]	ND	1.2 ± 0.1	ND	1.2 ± 0.1	3.9 ± 0.1	ND	ND	6.1 ± 0.1	ND	6.1 ± 0.2	4.2 ± 0.1
12 h	ND	ND	7.1 ± 0.1	1.7 ± 0.1	8.8 ± 0.1	6.4 ± 0.1	ND	ND	69.6 ± 0.3	1.3 ± 0.1	70.8 ± 1.2	6.9 ± 0.3
24 h	ND	ND	13.0 ± 0.2	1.3 ± 0.1	14.2 ± 0.1	6.9 ± 0.2	ND	0.4 ± 0	115.0 ± 2.5	8.1 ± 0.2	123.6 ± 2.3	7.1 ± 0.3
36 h	ND	ND	13.6 ± 0.2	4.2 ± 0.2	17.8 ± 0.1	7.0 ± 0.2	0.8 ± 0	1.6 ± 0.1	117.1 ± 2.2	15.0 ± 1.5	134.5 ± 3.5	7.3 ± 0.4
48 h	ND	1.1 ± 0.1	13.3 ± 0.4	6.4 ± 0.2	20.8 ± 0.3	6.6 ± 0.2	1.3 ± 0.1	4.1 ± 0.1	105.2 ± 1.4	23.4 ± 2.0	134.0 ± 3.1	7.1 ± 0.3

[a] Mevalonate was supplemented in culture at a concentration of 3.2 mM for synthesis of IPP and DMAPP using the bottom portion of a foreign MVA pathway. Geranoid production given as mg/L was presented as the mean of three replicated experiments. ND, not detected.

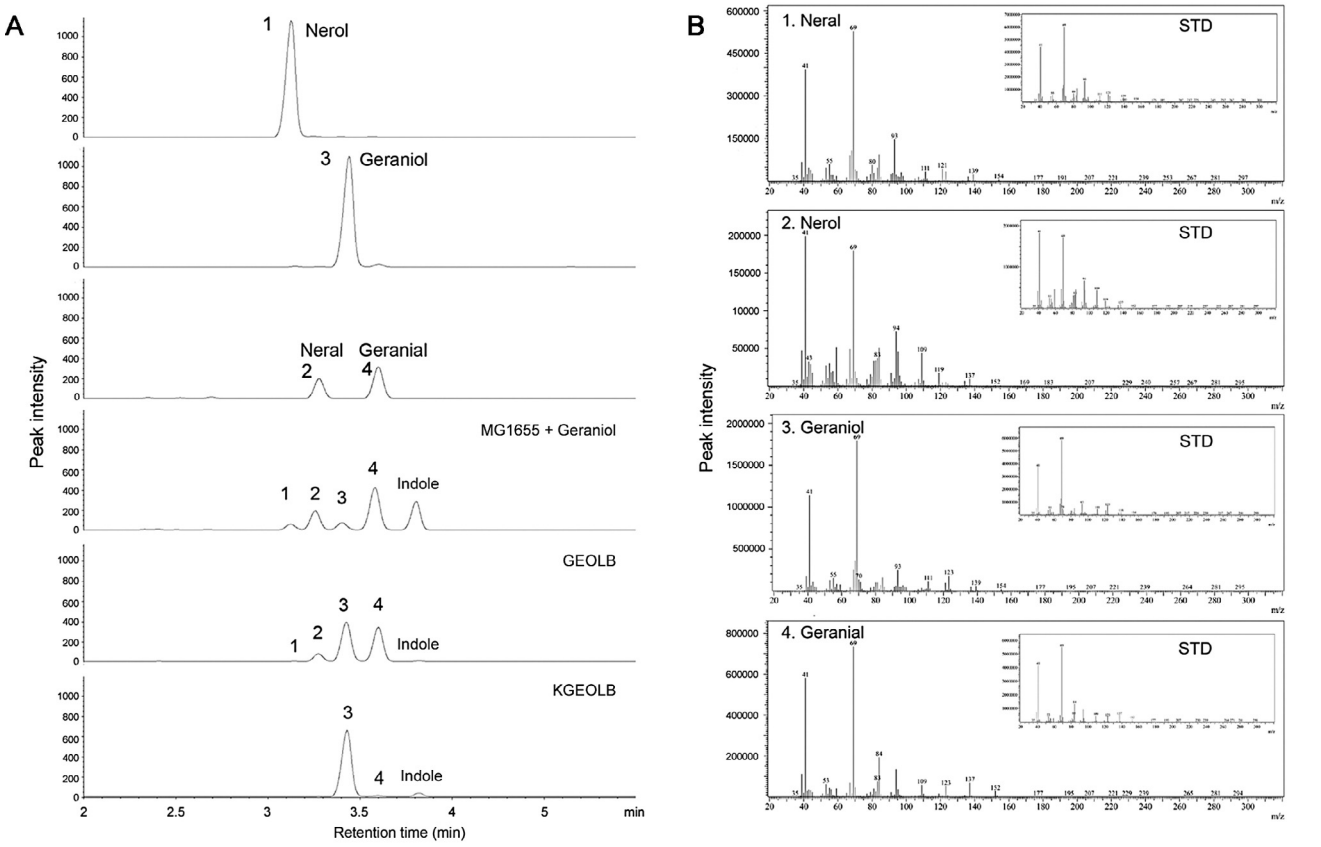

Fig. 2. GC and GC–MS profiles. (A) Top three panels represent standard compounds corresponding to the numbered peaks in the bottom panels. GC profiles of the decane phase from two-phase cultures of *E. coli* MG1655 with geraniol feeding, and *E. coli* GEOLB and KGEOLB are represented in the bottom three panels. Mevalonate was fed for the function of the bottom portion of MVA pathway in GEOLB and KGEOLB. (B) GC–MS chromatograms of the compounds indicated with numbered peaks in (A) and the corresponding standard compounds. Indole is a native compound produced from *E. coli*. The GC–MS chromatogram of the standard compound is shown in the upper right corner of each corresponding window. "STD" represents each standard compound.

3.3. Investigation of endogenous enzymes responsible for geranoid formation

Geraniol dehydrogenation in *O. basilicum* is naturally carried out by two enzymes, ObGEDH and ObCAD1 (Iijima et al., 2006). ObGEDH is a geraniol dehydrogenase with activities toward both geraniol and nerol, while ObCAD1 is known as a cinnamyl alcohol dehydrogenase with a considerable activity toward geraniol (Iijima et al., 2006). Three candidates (YjgB, YahK and YddN) in *E. coli* with high amino acid sequence identities to ObGEDH

Table 3
Time course analysis of geraniol oxidation and isomerization in *E. coli*.

Time (h)	Geraniol[a]	Nerol	Neral	Geranial	Total geranoids
12	167.7 ± 8.1	1.5 ± 0.2	1.3 ± 0.2	20.9 ± 1.3	191.4 ± 6.5
24	124.5 ± 11.2	6.5 ± 0.4	4.4 ± 0.2	46.3 ± 3.1	181.8 ± 13.1
48	56.9 ± 1.4	8.2 ± 0.9	31.1 ± 1.8	98.6 ± 7.8	194.9 ± 11.0
72	35.4 ± 1.1	8.3 ± 0.3	49.3 ± 2.4	92.8 ± 6.4	185.8 ± 9.1

[a] Geraniol was fed in about 200 mg/L in 5 ml culture broth of *E. coli* DH5α overlaid with 1 ml decane. The decane phases were collected at 12 h, 24 h, 48 h and 72 h, respectively. Results given as mg/L of each geranoid and total geranoids, respectively, are presented as the mean of three replicated experiments.

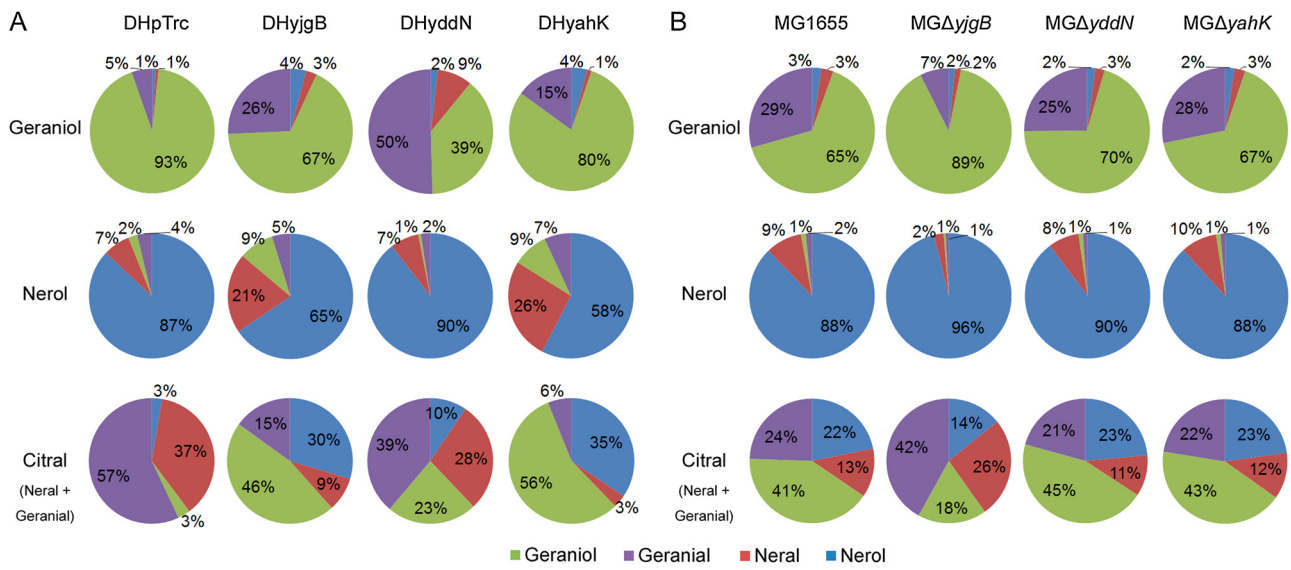

Fig. 3. Functional identification of geraniol dehydrogenase candidates using their over-expressing and knock-out strains based on geranoid feeding experiments. Geraniol, nerol or citral was fed at 200 mg/L in 5 ml cultures of DHpTr, DHyjgB, DHyddN and DHyahK (A), MG1655 and its knock-out mutants (MGΔ*yjgB*, MGΔ*yddN* and MGΔ*yahK*) (B). One milliliter of decane was initially overlaid for collecting geranoids. Seed was inoculated at a density of OD_{600}, 0.1. Cell cultures of DHpTr, DHyjgB, DHyddN and DHyahK were carried out at 30 °C and 250 rpm for 10 h with supplementation of 0.1 mM IPTG. Cell cultures of MG1655, MGΔ*yjgB*, MGΔ*yddN* and MGΔ*yahK* were carried out at 30 °C and 250 rpm for 24 h. The geranoids fed into the culture are represented on the left hand side. Assuming geranial and neral have the same GC sensitivity, the citral is a mixture of about 60% geranial and 40% neral. The compositional changes of geranoids from cell cultures are shown with a pie chart. Results are presented as the average value from three independent experiments.

and ObCAD1 were retrieved from NCBI BLAST search (Supporting information, Table S1). Among these candidates, YddN is known as a NADH-dependent medium-chain alcohol dehydrogenase/acetaldehyde reductase (Shafqat et al., 1999), whereas YjgB and YahK exhibit broad activities toward aldehydes and alcohols (Pick et al., 2013; Rodriguez and Atsumi, 2012). The highly conserved sequences of zinc-binding and NAD^+-binding sites in these geraniol dehydrogenase-like enzymes as compared to other alcohol dehydrogenases suggested that they belong to a medium-chain dehydrogenase/reductase (MDR) family (Jornvall et al., 1999) (Supporting information, Fig. S3). To investigate these candidates responsible for geraniol dehydrogenation, overexpression strains of DHyjgB, DHyddN and DHyahK were tested with geranoid feeding experiments. Strain DHpTrc harboring empty plasmid pTrc99A was used as a control. As expected, the overexpression of *yjgB*, *yddN* and *yahK* exhibited strong conversion activities to geraniol

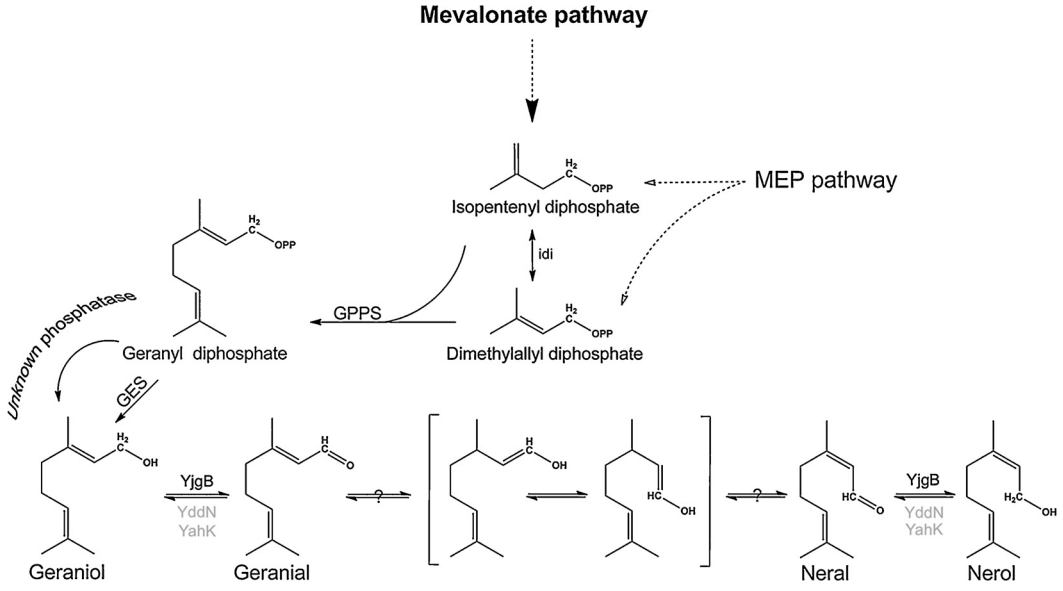

Fig. 4. A proposed pathway of geranoid formation in engineered *E. coli*. IPP and DMAPP generated from both the native MEP pathway and foreign MVA pathway are catalyzed by GPPS mutated from *E. coli* FPPS to make GPP. For geraniol formation, dephosphorylation of GPP can be catalyzed by either exogenous geraniol synthase (GES) or endogenous promiscuous phosphatases. In *E. coli*, geraniol can be converted into other geranoid derivatives including geranial, neral and nerol as shown here. Initially, geraniol is dehydrogenated into geranial by endogenous geraniol dehydrogenase-like enzymes such as YjgB, YddN and YahK. Geranial is then isomerized into neral via rotation of its C2—C3 bond, which is suspected to be a chemical keto-enol tautomerization (Iijima et al., 2006). Finally, neral is reduced to nerol by the geraniol dehydrogenase-like enzymes. Besides the inactivity of YddN toward nerol, all these enzymes can be expected to catalyze interconversion reactions between aldehyde forms and alcohol forms of geranoids. Among these enzymes, YjgB, indicated with black letters, plays a major role in the conversion of geranoids, whereas YddN and YahK, represented in gray letters, do not.

after 10 h of culture (Fig. 3A), although the cell growth was somewhat affected by their overexpression as compared with DHpTrc (Supporting information, Fig. S4A). The DHyjgB, DHyddN and DHyahK strains converted 33%, 61% and 20% of fed geraniol into other geranoids, respectively, which were much higher than that of the control strain (7%). Besides, these overexpression strains also showed significant conversion activities toward the fed nerol and citral (a mixture of about 40% neral and 60% geranial) over the control strain. The three genes were also deleted in MG1655 to create their mutant strains, MGΔ*yjgB*, MGΔ*yddN* and MGΔ*yahK* (Table 1) for identification of their specificities to geraniol dehydrogenation. The genes deletion did not affect the cell growth of the strains (Supporting information, Fig. S4B), while different conversion patterns of fed geranoids were observed among these mutants (Fig. 3B). Wild type strain MG1655 converted 35% of the fed geraniol into geranial (29%), neral (3%), and nerol (3%), respectively; the same conversion patterns were observed in the MGΔ*yddN* and MGΔ*yahK* strains; however, in the MGΔ*yjgB* strain, conversion of geraniol to other geranoids was significantly reduced to 11%. Similar patterns were observed when feeding nerol and citral, whereby the MGΔydd*N* and MGΔ*yahK* strains exhibited the same conversion pattern as the wild type strain; in the MGΔ*yjgB* strain, conversion of fed nerol to other geranoids was reduced from 12% to 4%, and conversion of neral and geranial to other geranoids during the citral feeding experiment was also reduced by almost 50% in both cases. The above results collectively suggested that YjgB was the major one responsible for the endogenous formation of geranoids during geraniol production, whereas YddN and YahK were not significantly involved due to their natively suppressed expression.

As the geraniol, geranial, neral and nerol formations were sequentially observed during culture (Table 2), we inferred a mechanism for geranoid formation in *E. coli*: nerol is generated from the reduction of neral, while neral is formed via the isomerization of geranial, and geranial is produced through the dehydrogenation of geraniol, which is consistent with the geranoid formation mechanism in the glands of sweet basil, as proposed by Iijima et al. (2006). The proposed metabolic pathway of geranoid formation during geraniol synthesis in *E. coli* is depicted in Fig. 4.

3.4. Optimization of geraniol production via prevention of geraniol dehydrogenation

Since geraniol production encountered a loss from its endogenous dehydrogenation mediated by YjgB in *E. coli*, the *yjgB* knock-out strain (KGEOLB) was established to increase geraniol production and its purity (Table 1). After 48 h of culture, geraniol production was improved from 105.2 mg/L in GEOLB to 129.7 mg/L in KGEOLB, while the purity of geraniol in geranoids was increased from 78% to 98% (Fig. 5A). It was found that deletion of gene *yjgB* in strain KGEOLB resulted in declined cell growth as compared to

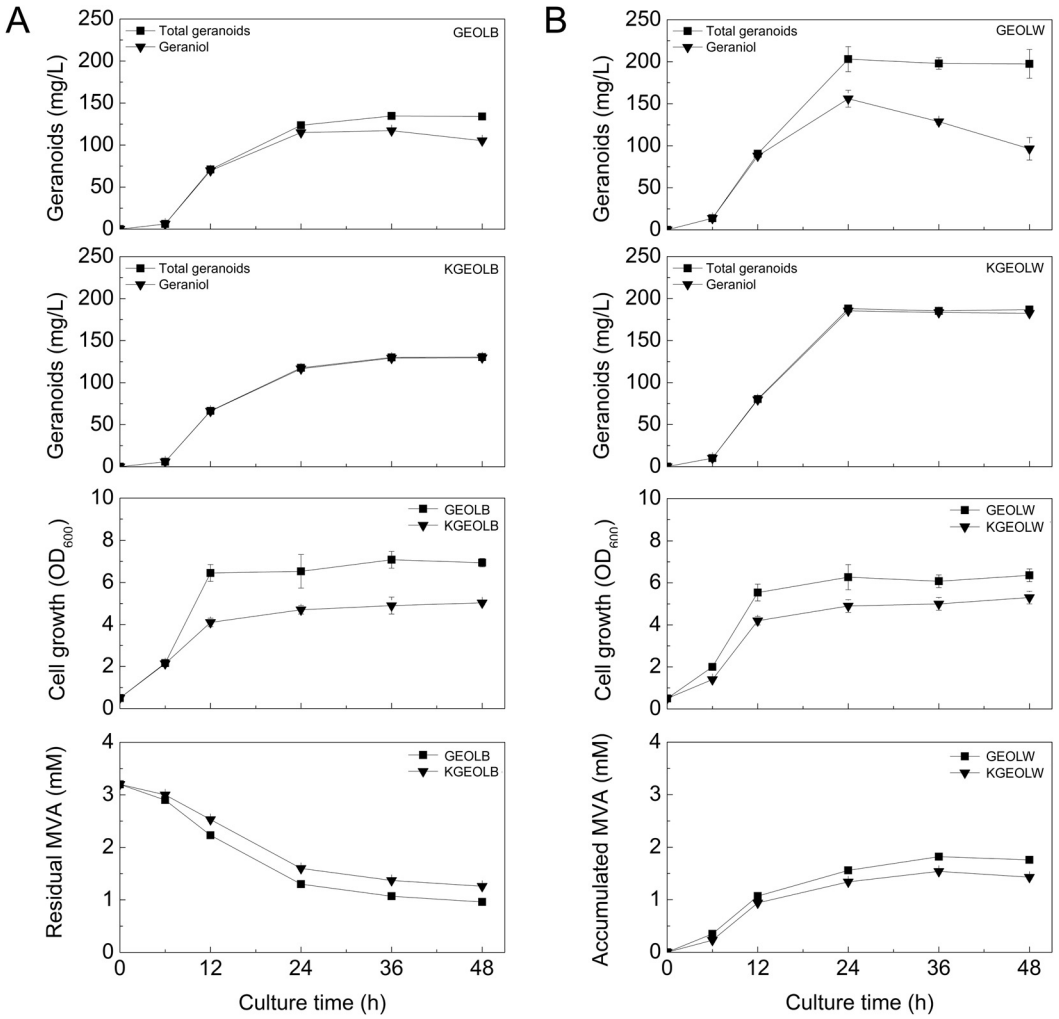

Fig. 5. Effect of *yjgB* knockout on geranoid production and cell growth. (A) Geraniol production from strains GEOLB and KGEOLB harboring geraniol synthesis operon and bottom portion of MVA pathway with supplementation of 3.2 mM MVA. (B) Geraniol production from strains GEOLW and KGEOLW harboring geraniol synthesis operon and whole MVA pathway. Results are the mean of three replicates with error bars representing the standard deviation.

the control strain GEOLB (Fig. 5A). It was plausible that the declined cell growth was caused by the higher accumulation of toxic geraniol in strain KGEOLB (25.9 mg/L/OD_{600}) compared to strain GEOLB (19.3 mg/L/OD_{600}). However, under the decane overlay two-phase culture system, the intracellular toxicity arisen from the accumulation of geraniol would not be critical because most of geraniol could be extracted into the decane phase, and 200 mg/L of geraniol did not affect the cell growth of *E. coli* MGΔ*yjgB* from the feeding experiment (Supplementation information, Fig. S5). The mechanism of *yjgB* deficiency affecting the cell growth of KGEOLB remains to be elucidated.

3.5. Improved geraniol production using the whole MVA pathway in yjgB deletion strain

A large residual amount of fed mevalonate (about 35% of the feeding amount) was observed in culture broth during geraniol production from the bottom portion of MVA pathway (Fig. 5A), which was similar to the situation encountered by Wang et al. during the production of α-farnesene from the bottom portion of MVA pathway (Wang et al., 2011). The inefficient consumption of fed mevalonate was suspected to be a result of the mevalonate uptake inefficiency of host cells, since much more improved α-farnesene production was obtained by producing mevalonate endogenously using the whole MVA pathway from a cheap carbon source in *E. coli* (Wang et al., 2011). In the present study, it was also investigated whether geraniol production could be improved by introducing the whole MVA pathway for endogenously biosynthesizing mevalonate from a cheap carbon source. Thus the bottom portion of MVA pathway (pSTKSN12Didi) was replaced in strains GEOLB and KGEOLB with the whole MVA pathway (pSNAK) forming strains GEOLW and KGEOLW, respectively. After 48 h of culture, the titers of total geranoid production in both strains were increased to 197.5 mg/L and 187.0 mg/L, respectively (Fig. 5B). However, geraniol production in strain GEOLW encountered a loss of 101.0 mg/L (52%) after 48 h of culture, which was much higher than that of 28.8 mg/L in strain GEOLB (22%) (Fig. 5). As the accumulation of total geranoids and the cell growth of strains GEOLW and GEOLB were ceased after 36 h of culture, geraniol dehydrogenation rate of both strains was compared during the cultures between 36 h and 48 h. The geraniol dehydrogenation rate in strain GEOLW (0.43 mg/L/h/OD_{600}) was turned out to be 3-fold of that in strain GEOLB (0.14 mg/L/h/OD_{600}), which suggested that a higher production of geraniol would elicit its faster dehydrogenation in a strain bearing YjgB, and elimination of *yjgB* in *E. coli* is crucial for producing geraniol at a high titer. Therefore, by both introduction of the whole MVA pathway and deletion of *yjgB* gene, geraniol production was ultimately doubled from 96.5 mg/L in strain GEOLW to 182.5 mg/L in strain KGEOLW after 48 h of culture (Fig. 5B).

4. Conclusions

In summary, this work demonstrated an engineering process for improved geraniol production in *E. coli*. Our results indicated that the endogenous dehydrogenation of geraniol in host was the major factor hindering geraniol production as the building blocks were adequately supplied. We preliminarily identified the candidate enzymes in *E. coli* responsible for the endogenous dehydrogenation of geraniol based on the high amino acid sequence identities between plant geraniol dehydrogenases and *E. coli* proteins. By knockout of the primary gene (*yjgB*) responsible for the endogenous dehydrogenation of geraniol, and introduction of an efficient foreign MVA pathway, geraniol production was finally produced to a level of 182.5 mg/L in 48 h. So far, it is the highest level of geraniol production in engineered microorganisms, and is approximately 5-fold of the highest production reported before (36 mg/L) (Liu et al., 2013). The approach applied for the selective geraniol production by identifying and removing the endogenous promiscuous metabolic activity interfering foreign metabolic product formation should be a good reference for addressing similar problems. Recently, Alonso-Gutierrez et al. successfully produced L-limonene, another monoterpene, to a level of 430 mg/L in engineered *E. coli* with a heterologous MVA pathway by carefully balancing the L-limonene biosynthesis pathway (Alonso-Gutierrez et al., 2013). Therefore, a great improvement in geraniol production is expected as the geraniol biosynthesis pathway can be further balanced in the mutant strain KGEOLW by refereeing to the approaches used for L-limonene production (Alonso-Gutierrez et al., 2013).

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jbiotec.2013.11.009.

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