Biosynthesis of geranate via isopentenol utilization pathway in Escherichia coli

Qiuchi Pan[1], Xiaoqiang Ma[1], Hong Liang[1], Yurou Liu[1], Greg Stephanopoulos[1], and Kang Zhou[1]

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Abstract

Isoprenoids are a large family of natural products with diverse structures, which allow them to play diverse and important roles in the physiology of plants and animals. They also have important commercial uses as pharmaceuticals, flavouring agents, fragrances, and nutritional supplements. Recently, metabolic engineering has been intensively investigated and emerged as the technology of choice for the production of isoprenoids through microbial fermentation. Isoprenoid biosynthesis typically originates in plants from acetyl-coA in central carbon metabolism, however, a recent study reported an alternative pathway, the Isopentenol Utilization pathway (IUP), that can provide the building blocks of isoprenoid biosynthesis from affordable C5 substrates. In this work, we expressed the IUP in *Escherichia coli* to efficiently convert isopentenols into geranate, a valuable isoprenoid compound. We first established a geraniol-producing strain in *E. coli* that uses the IUP. Then, we extended the geraniol synthesis pathway to produce geranate through two oxidation reactions catalysed by two alcohol/aldehyde dehydrogenases from *Castellaniella defragrans*. The geranate titer was further increased by optimizing the expression of the two dehydrogenases and also parameters of the fermentation process. The best strain produced 764 mg/L geranate in 24 h from 2 g/L isopentenols (a mixture of isoprenol and prenol). We also investigated if the dehydrogenases could accept other isoprenoid alcohols as substrates.

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ABSTRACT

Isoprenoids are a large family of natural products with diverse structures, which allow them to play diverse and important roles in the physiology of plants and animals. They also have important commercial uses as pharmaceuticals, flavouring agents, fragrances, and nutritional supplements. Recently, metabolic engineering has been intensively investigated and emerged as the technology of choice for the production of isoprenoids through microbial fermentation. Isoprenoid biosynthesis typically originates in plants from acetyl-coA in central carbon metabolism, however, a recent study reported an alternative pathway, the Isopentenol Utilization pathway (IUP), that can provide the building blocks of isoprenoid biosynthesis from affordable C5 substrates. In this work, we expressed the IUP in *Escherichia coli* to efficiently convert isopentenols into geranate, a valuable isoprenoid compound. We first established a geraniol-producing strain in *E. coli* that uses the IUP. Then, we extended the geraniol synthesis pathway to produce geranate through two oxidation reactions catalysed by two alcohol/aldehyde dehydrogenases from *Castellaniella defragrans* . The geranate titer was further increased by optimizing the expression of the two dehydrogenases and also parameters of the fermentation process. The best strain produced 764 mg/L geranate in 24 h from 2 g/L isopentenols (a mixture of isoprenol and prenol). We also investigated if the dehydrogenases could accept other isoprenoid alcohols as substrates.

INTRODUCTION

The isoprenoid family comprises more than 65,000 compounds (Berthelot et al., 2012) that have found many useful applications in the manufacturing of drugs, fragrances, food additives, colorants, and rubber and advanced biofuels (Gershenzon & Dudareva, 2007). Currently, these compounds are produced for commercial use by extraction from plants (Daletos et al., 2020). In order to satisfy increasing market demand and reduce production cost, microbial production of isoprenoids is investigated (Chang & Keasling, 2006). Metabolic engineering has enabled the construction of strains with attractive properties for isoprenoid production in microbial hosts (Li et al., 2020; Schempp et al., 2018).

Nearly all isoprenoids are synthesized from two precursors, isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP). These precursors are biosynthesized in nature by two distinct metabolic pathways, the methylerythritol phosphate (MEP) pathway, which is present in most bacteria and plastids of plant cells, and the mevalonate (MVA) pathway, which functions in most eukaryotes, archaea, and certain bacteria. IPP and DMAPP are subsequently condensed to generate geranyl diphosphate (GPP), farnesyl diphosphate (FPP) and geranylgeranyl diphosphate (GGPP). These linear diphosphate intermediates can be further functionalized into various structures and the diversity of these reactions is responsible for the synthesis of many diverse isoprenoid compounds (Daletos et al., 2020). The IPP and DMAPP precursors of the two native pathways are derived from central metabolism, requiring many reaction steps for the conversion of a typical carbon substrate like glucose to an isoprenoid molecule. These pathways also compete with cell biosynthesis for building blocks and, as such, are subject to native host regulation.

To overcome the above problems, a novel pathway (IUP) was recently proposed whereby the two key precursors, IPP and DMAPP, are synthesized from isopentenols by only two consecutive phosphorylation steps (Chatzivasileiou et al., 2019). IUP comprises only two enzymes that sequentially phosphorylate isoprenol (or prenol) into isopentenyl phosphate (or dimethylallyl phosphate), and then into IPP (or DMAPP). DMAPP and IPP are isomerized into each other by IPP isomerase (IDI) to balance their ratio. Compared with the MVA and MEP pathways, IUP does not require any building blocks from central metabolism and is less energetically demanding (Liu et al., 2020; Luo et al., 2020; Ward et al., 2019).

Geranate is a valuable C10 isoprenoid compound with broad industrial applications. Geranate can be used as a perfuming agent in cosmetics (Mi et al., 2014) and has also been identified as a superior antifungal agent against two main phytopathogens of corn, *Colletotrichum graminicola* and *Fusarium graminearum* . As such, it was recently produced in a transgenic maize plant to control fungal disease outbreak (Yang et

al., 2011). Geranate also has potential as insecticide since it possesses excellent insecticidal activity against *Stephanitis pyrioides* and *Aedes aegypti* as well as high biting deterrent activity (Ali et al., 2013). Moreover, geranate is known to be a tyrosinase inhibitor and inhibits melanin synthesis (Wang & Hebert, 2006) in applications of skin depigmentation. However, the production of geranate by engineered microbes has not been systematically pursued.

Geranate could be obtained from the oxidation of geraniol, a commercially important fragrance molecule (Chen & Viljoen, 2010). Geraniol in turn can be synthesized from GPP and has been produced from isopentenols and other simple substrates through microbial fermentation. Geraniol is formed in *C. defragrans* cells during its growth onβ -myrcene via hydration and isomerization (Brodkorb et al., 2010). Geranate is also identified as an intermediate in this culture, and the relevant proteins (geraniol dehydrogenase [CdGeDH] and geranial dehydrogenase [CdGaDH]) in *C. defragrans* were purified and sequenced (Luddeke et al., 2012).

In this work, we first established a geraniol production pathway which produces 750 mg/L geraniol from 2 g/L isopentenols in 24 h. Then we extended the geraniol production pathway to produce geranate through two oxidation reactions catalysed by CdGeDH and CdGaDH. After optimizing the expression level of CdGeDH and CdGaDH, the engineered *E. coli*strain could produce up to 764 mg/L geranate from 2 g/L isopentenols within 24 h. We also confirmed that producing geranate did not require an organic overlay because of its high water-solubility. The use of organic overlays complicates production and downstream processing and increases the product purification cost. As such, processes that do not require such organic overlays for the isoprenoid production are advantageous.

Additionally, we found that CdGeDH and CdGaDH can oxidize various C5 to C15 isoprenoid alcohols. The geranate-producing strain developed in this study provides a promising basal strain for developing manufacturing processes for the production of geranate and its derivatives.

METHODS

Chemicals

All chemicals were purchased from Sigma-Aldrich unless otherwise specifically mentioned. Synthetic DNA and oligos were synthesized by Integrated DNA Technologies.

Plasmids and strains construction

All plasmids constructed in the current study were conducted by using Guanin/Thymine standard (Ma et al., 2019). Constructed plasmids were verified by Sanger sequencing (service provider: Bio Basic Asia Pacific Pte Ltd, Singapore). Each of the constructed plasmids was introduced into *E. coli* **MΓ1655_ΔE3 (*Δρϵς A*, *Δϵνδ A*, ΔE3)** via standard electroporation protocol (Ma et al., 2019). Successfully constructed strains were isolated on Luria Bertani (LB) agar (BD, DIFCO) plate containing proper antibiotics. All plasmids and strains used in this study are summarized in **Table 1** .

Table 1 . Plasmids and strains used in this study.

Strains	Plasmids
GE01	pIG_01 (P_{BAD}_EcthiM_MvIPK_Ecidi_P_{T7}_ObgeS_Aggpps_Amp^R_pMB1)
GA01	pIG_01
	pGA_01 (P_{cymO}_CdGeDH_CdGaDH_$Spect^R$_pAC)
GA02	pIG_01
	pGA_02 (P_{cymO}_CdGeDH_CdGaDH_$Spect^R$_pSC101)
GA03	pIG_01
	pGA_03 (P_{T7}_CdGeDH_CdGaDH_$Spect^R$_pSC101)
GA04	pIG_01

Strains	Plasmids
	pGA_04 (P_{thrC3}_CdGeDH_CdGaDH_$Spect^R$_pSC101)
BGA01	pGA_01

E. coli **MΓ1655_ΔρεϛA_ΔενδA_ΔE3** was used as the parent strain. $Spect^R$: spectinomycin resistance. Amp^R: ampicillin resistance.

Cell culture and media

Seed cultures were started in 14 mL round-bottom falcon tubes, which were incubated at 37 °C/250 rpm overnight. Cell density was measured by Varioskan LUX multimode microplate reader (Thermo Fisher Scientific) at the wavelength of 600 nm. The overnight grown seed culture was used to inoculate (1.5%, v/v) 25 mL of K3 medium (Zhou et al., 2015) in 250 mL shake flasks. Subsequent cell culture processes were conducted at 30 °C/250 rpm. The K3 medium composition was as follows (working concentration): 10 g/L glucose, 10 g/L tryptone, 5 g/L yeast extract, 13.3 g/L KH_2PO_4, 4 g/L $(NH_4)_2HPO_4$, 0.0084 g/L EDTA, 0.0025 g/L $CoCl_2$, 0.015 g/L $MnCl_2$, 0.0015 g/L $CuCl_2$, 0.003 g/L H_3BO_3, 0.0025 g/L Na_2MoO_4, 0.008 g/L Zn $(CH_3COO)_2$, 0.06 g/L Fe(III) citrate and 1.3 g/L $MgSO_4$. Antibiotics (100 μg/mL ampicillin and/or 50 μg/mL spectinomycin) were added according to **Table 1** , and pH was adjusted to 7 using 400 g/L sodium hydroxide solution. Inducers were added when cell density (OD_{600}) reached ˜0.5 to trigger expression. 0.01 mM IPTG, 4 g/L L-arabinose and 0.03 mM cumic acid were added at this stage when gene expression was controlled by P_{T7}, $P_{BAD}$and P_{cymO}, respectively. Cells were collected 6 hours after induction.

The grown cells were centrifuged at 4,000 g for 8 min. Cell pellets were resuspended in 5 mL of fresh K3 medium to a final OD of 20. In the fresh K3 medium, 20 g/L L-arabinose, 10 g/L tryptone and 5 g/L yeast extract were used as carbon sources. 0.05 mM IPTG or 0.03 mM cumic acid was used in the second gene expression stage using P_{T7} or P_{cymO}. One millilitre of re-suspended cells was transferred to a 14 mL round-bottom falcon tube. A total of 2 g/L isoprenol and prenol at a molar ratio of 3:1 was supplemented as substrate. A 15% v/v hexadecane layer was used under same conditions (as specified in **Results and Discussion**). The culture was incubated at 30 °C/250 rpm for 24 h. For the biotransformation of nerol, perillyl alcohol and farnesol, 2 g/L of nerol, perillyl alcohol and farnesol were added instead of isoprenol and prenol.

Extraction and quantification of geraniol and geranate in organic phase

Two hundred millilitres of cell culture (including organic layer) were collected and transferred to a 1.7 mL centrifuge tube (Axygen) followed by centrifugation at 10,000 g for 10 min. Two microliters of the hexadecane phase was taken and diluted with 98 μL ethyl acetate (EA). One microliter of diluted sample was analyzed by gas chromatography-mass spectrometry (GC/MS, 5977B, Agilent Technologies) to quantify geraniol and geranate in the hexadecane phase. HP-5MS capillary column (30 m × 0.25 mm, 0.25 μm film thickness, Agilent Technologies) was used, with helium as the carrier gas. The following oven temperature program was carried out: 50 °C for 1 min, 50–100 °C at a rate of 5 °C/min, 100 °C for 1 min, 100–200 °C at a rate of 50 °C/min, 200 °C for 1 min, 200–300 °C at a rate of 50 °C/min and 300 °C for 1 min. The reported geraniol and geranate titer is the sum of the products in the hexadecane phase and aqueous phase when two phases were used.

Extraction and quantification of isopentenols and geraniol in aqueous phase Two hundred millilitres of cell culture (including organic layer) were collected and transferred to a 1.7 mL centrifuge tube (Axygen) followed by centrifugation at 10,000 g for 10 min. One hundred microliters of supernatant was mixed with one hundred microliters of EA and the mixture was vortexed for 30 min. The solution was then centrifuged at 10,000 g for 10 min. The EA phase (1 μL) was analyzed by GC/MS to quantify geraniol and isopentenols in the aqueous phase. The same GC/MS method was used as described above.

Extraction and quantification of geranate, 3-methyl-crotonate and 3-methyl-3-butanoate in aqueous phase

One hundred microliters of cell culture were collected and centrifuged at 10,000 g for 10 min. Four hundred microliters of methanol was mixed with the obtained supernatant and vortexed for 1 h. The mixture was then centrifuged at 10,000 g for 10 min to remove the precipitated enzymes and the supernatant was then analysed. An Ultra Performance Liquid Chromatography (UPLC, Waters ACQUITY) linked with a Time-of-flight Mass Spectrometry (TOFMS, Bruker micrOTOF II) was used to quantify geranate, 3-methyl-crotonate (3-MC) and 3-methyl-3-butanoate (3-MB). The column used was Poroshell 120 EC-C18 column (2.1 × 50 mm, 2.7 μm, Agilent Technologies). Two mobile phases were used: A) 0.1 % trifluoroacetic acid in water; B) acetonitrile. The gradient was as follows: 0–2 min, 95% A; 2-2.5 min, 95%-90% A; 2.5-3.5 min, 90% A, 3.5-5.5 min, 90%-60% A, 5.5-9 min, 60% A; 9-10 min, 60%-95% A; 10-11 min, 95% A. The flow rate was 0.25 mL/min. Electrospray ionization was used and the mass spectrometry was operated to scan 50–800 m/z in positive mode with 2,500 V end plate voltage and 3,800 V capillary voltage. Nebulizer gas was provided in 1 bar, drying gas temperature was 9 mL/min, and dry gas temperature was 200 °C. Sample injection volume was 10 μL.

RESULTS AND DISCUSSION

Engineering *E. coli* to produce geraniol from isopentenols

We first engineered the IUP in *E. coli* strains for the heterologous production of geraniol (**Figure 1**). In prior work we had found that using *E. coli* hydroxyethylthiazole kinase (EcthiM) (Clomburg et al., 2019) and *Methanococcus vannieliii*isopentenyl phosphate kinase (MvIPK) (Couillaud et al., 2019) yielded higher lycopene titers relatively to other combinations of IUP kinases tested (Ma et al., 2022). Thus, we used *EcthiM* and *MvIPK*in this study too for the biosynthesis of IPP and DMAPP as precursors for geraniol synthesis. We also overexpressed *E. coli idi*to better balance the ratio of DMAPP to IPP, and geranyl diphosphate synthase (Aggpps) from *Abies grandis* to catalyse the condensation of DMAPP and IPP into GPP (Burke & Croteau, 2002). The gene of a truncated geraniol synthase (Obges) from sweet basil *Ocimum basilicum* was overexpressed for converting the intermediate GPP into geraniol (Iijima et al., 2004). Genes involved in IUP (*EcthiM, MvIPK* and *Ecidi*) were co-expressed in one operon under the control of an inducible promoter (P_{BAD}). Downstream genes for geraniol production (*Aggpps* and *Obges*) were co-expressed in another operon driven by another inducible promoter (P_{T7}). These two operons were placed in one plasmid (**Figure 2a**). The resulting geraniol-producing strain was named**GE01** . Strain and plasmid information are included in

Table 1.

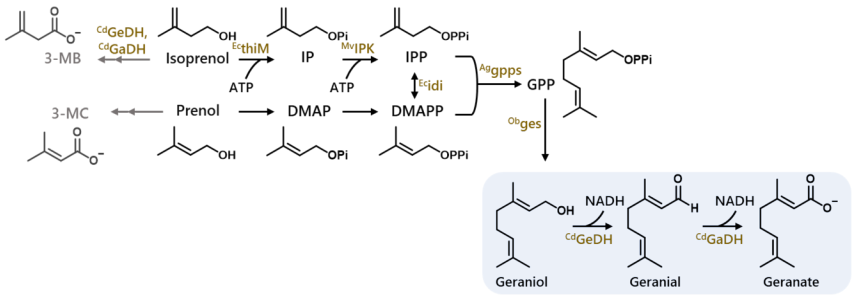

Figure 1. The metabolic pathway to synthesize geraniol and geranate from isoprenol/prenol. The host organism used in this study was*E. coli* . IP: Isopentenyl monophosphate; IPP: Isopentenyl diphosphate; DMAP: Dimethylallyl monophosphate; DMAPP: Isomer dimethylallyl diphosphate; GPP: Geranyl diphosphate; 3-MC: 3-Methyl-crotonate; 3-MB: 3-Methyl-3-butanoate. EcthiM: *E. coli*hydroxyethylthiazole kinase; MvIPK: *Methanococcus vannieliii*isopentenyl phosphate kinase; Ecidi: *E. coli* IPP isomerase; Aggpps: *Abies*

grandis geranyl diphosphate synthase; Obges: *Ocimum basilicum* geraniol synthase; CdGeDH: *Castellaniella defragrans* geraniol dehydrogenase; CdGaDH: *Castellaniella defragrans* geranial dehydrogenase.

We adopted a two-stage fermentation protocol for isoprenoid production (Ma et al., 2022). In brief, the first stage is for cell growth and gene expression. The engineered strains were cultured in a medium containing glucose, tryptone and yeast extract with the goal to accumulate cell biomass. Six hours after induction, cells were collected for use in the second stage fermentation. In this stage, cells were re-suspended in fresh media (OD_{600} = 20) containing 2 g/L isopentenols (1.5 g/L isoprenol and 0.5 g/L prenol). 15% (v/v) of hexadecane was added as organic overlay to continuously extract the produced geraniol. The reported geraniol titer is the sum of the geraniol in the aqueous and organic phases. **GE01** consumed 1.5 g/L isopentenols (**Figure 2e**) and produced 750 mg/L geraniol within 24 h in the second stage (**Figure 2c**). The geraniol-producing strain and two-stage fermentation protocol were used in the subsequent experiments.

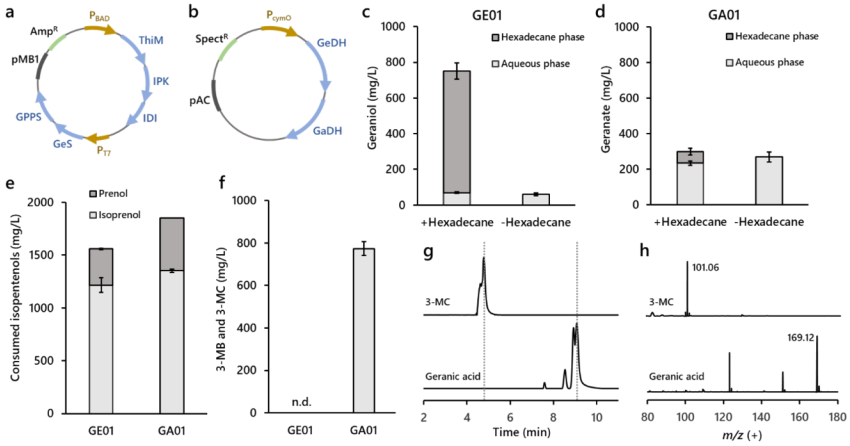

Figure 2. a) Illustration of the plasmid used in geraniol production in **GE01** (*pIG_01*). **b)**Illustration of the plasmid used in converting geraniol into geranate in**GA01** (*pGA_01*). Production of **c)** geraniol with**GE01** and **d)** geranate with **GA01** with and without hexadecane within 24 h. No geranate was detected in the culture of **GE01** and no geraniol was detected in the culture of**GA01.** **e)** Isopentenol consumption by **GE01** or**GA01** within 24 h with hexadecane. **f)** Formation of by-products (3-MC and 3-MB) by **GE01** or **GA01** within 24 h. n.d., not detected. **g)** Extracted ion chromatogram (EIC) of 3-MC (m/z=101.06) and geranic acid (m/z=169.12) standards. 3-MC was eluted at 4.8 min and geranic acid was eluted at 9.2 min. **h**) mass spectra of 3-MC and geranic acid. Error bars indicate standard error (n=3).

Pathway construction for conversion of geraniol into geranate in *E. coli*

To convert geraniol into geranate, we co-expressed *CdGeDH* and*CdGaDH* from *C. defragrans* in one operon in a separate plasmid (*pGA01*) under the control of a strong inducible promoter P_{cymO} (**Figure 2b**). We named the geranate production strain **GA01** . After the fermentation, Liquid Chromatography Mass Spectrometry (LC/MS) was used to quantify geranate in the aqueous phase (**Figure 2g, h**); Gas Chromatography Mass Spectrometry (GC/MS) was used to quantify geranic acid in the organic overlay. The fermentation produced 298 mg/L geranate with **GA01**(**Figure 2d**). Geraniol and geranial were not detected in the culture of **GA01** , indicating that CdGeDH and CdGaDH have high catalytic efficiency with geraniol and geranial, respectively.

While geraniol was partitioned more favourably into the organic phase of the **GE01** culture (**Figure 2c**), most of the geranate accumulated in the aqueous phase of the **GA01** culture (**Figure 2d**). This was expected because geraniol (LogP = 3.56) (Griffin et al., 1999) is more hydrophobic than geranate

(LogP = 2.8) (Ko et al., 2021). Hexadecane is frequently used to prevent loss of volatile products (such as geraniol). Using hexadecane, however, complicates downstream product purification (it is challenging to separate geraniol from hexadecane). When we removed the hexadecane from the **GE01** culture, the geraniol titer was reduced to 8% of the control (with hexadecane, **Figure 2c**). We, however, found that a similar geranate titer (268 mg/L) was achieved with or without hexadecane (**Figure 2d**). Hexadecane was not used in the rest of this study to simplify the fermentation process.

Next, we determined the consumption of isopentenols in the cultures, and found that **GA01** consumed more isopentenols than **GE01**(**Figure 2e**). Since the titer of geranate in the **GA01**culture was much lower than that of geraniol in the **GE01**culture (**Figure 2c,d**), this was an indication that by-products were formed along with geranate by the geranate-producing **GA01** . We hypothesized that the hydroxyl group of isoprenol and prenol may also be oxidized by CdGeDH and CdGaDH to form corresponding carboxylates (3-methyl-crotonate [3-MC] and 3-methyl-3-butanoate [3-MB],**Figure 1**), as these oxidation reactions would compete with the downstream reaction of geranate biosynthesis. We confirmed this hypothesis by analysing the aqueous phase of **GE01** and**GA01** cultures with LC/MS (**Figure 2f**). As shown in**Figure 2f** , 773 mg/L of by-products (3-MC and 3-MB) were produced by **GA01** while no by-products were found in the culture of **GE01** (**Figure 2f**).

Tuning expression of CdGeDH and CdGaDH to reduce by-product formation

It is possible that the expression level of CdGeDH and CdGaDH was too high in **GA01** , rapidly oxidizing a large amount of isopentenols into the by-products and competing with EcthiM (the first kinase in IUP) for substrate. This suggests a reduction in the expression level of CdGeDH and CdGaDH so that more isopentenols could enter the IUP for production of geranate. To test this hypothesis, we constructed a new plasmid (*pGA02*) with a low copy number replication origin (pSC101; copy number: ˜5) to weaken the expression of CdGeDH and CdGaDH (*pGA01* used pAC replication origin; copy number: 10-15). We replaced *pGA01* with *pGA02* in**GA01** , creating a new strain (**GA02**). The geranate produced by **GA02** (764 mg/L) was almost three times that produced by **GA01** (**Figure 3a**), and the concentration of the by-products (3-MC and 3-MB) was substantially reduced (from 865 mg/L to 436 mg/L) (**Figure 3b**). Then we replaced the P_{cymO} promoter in **GA02** with P_{T7}or P_{thrC3} (Anilionyte et al., 2018), creating *E. coli* **GA03** and **GA04** , respectively. These two strains produced a similar amount of geranate as **GA02** (**Figure 3a**), but with significantly reduced amount of by-products (68 mg/L and 190 mg/L 3-MC/3-MB by **GA03** and **GA04** , respectively,**Figure 3b**). A low IPTG concentration (0.05 mM) was used in this study, so P_{T7} could be weaker than P_{thrC3} and P_{cymO}.

A previous study successfully engineered *Acinetobacter sp. Tol 5*to transform geraniol into geranate (Usami et al., 2020). Their geranate titer was similar to what we achieved in this study. Our process, however, used a cheaper substrate (isopentenols instead of geraniol) and was much faster (24 h instead of 144 h).

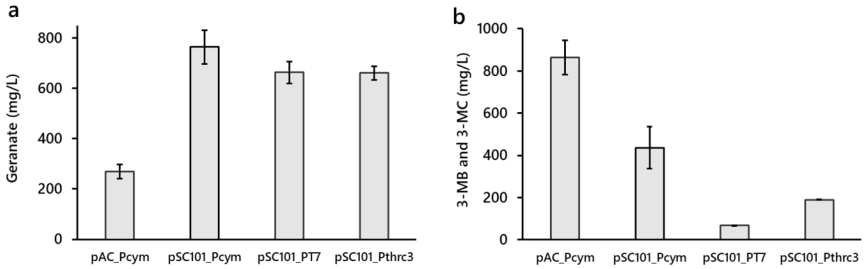

Figure 3. Controlled expression of the geraniol dehydrogenase and geranial dehydrogenase to reduce by-product formation. **a)**Geranate titer of **GA01** , **GA02** , **GA03** and**GA04** (from left to right) at 24 h (2 g/L

isopentenols).**b)** 3-MC/3-MB concentration of the geranate-producing strains at 24 h. Error bars indicate standard error (n=3).

Testing CdGeDH and CdGaDH with other isoprenoid alcohols

We further examined whether CdGeDH and CdGaDH could oxidize other isoprenoid alcohols into corresponding aldehydes and acids. We selected three natural isoprenoid alcohols, nerol (C10), perillyl alcohol (C10) and farnesol (C15, **Figure 4a**) with the expectation that the hydroxyl groups of those compounds would also be oxidized by CdGeDH and CdGaDH into aldehyde or carboxylate group (**Figure 4a**). In this case, nerol, perillyl alcohol and farnesol would be oxidized into neral, perillyl aldehyde and farnesal respectively and then would be further oxidized into nerolic acid, perillic acid and farnesoic acid. Nerolic acid is identified as a nassanoff pheromone of the honey bee (Boch & Shearer, 1964). Perillic acid is an antineoplastic agent (Crowell et al., 1992). Farnesoic acid is a key precursor for the biosynthesis of C16 juvenile hormone which plays key roles in both metamorphosis and adult reproductive processes (Liu et al., 2010).

We designed a new strain without the geraniol-producing plasmid (**BGA01**) for the conversion of nerol, perillyl alcohol and farnesol. We cultured **BGA01** with 2 g/L of nerol, perillyl alcohol or farnesol with the addition of hexadecane overlay (to reduce alcohol evaporation). Then we monitored substrate consumption and product formation in both aqueous and hexadecane phases with GC/MS. When perillyl alcohol or nerol were supplemented, all added amounts were consumed by **BGA01** within 24 h. We could observe clear peaks for their corresponding carboxylic acids (perillic acid and nerolic acid) in both phases by using GC/MS (**Figure 4b**). As a control, the parent strain of BGA01 did not form any of these products.*Pseudomonas putida DSM 12264* was used to transform limonene into perillic acid via perillyl alcohol (Mirata et al., 2009). The genes responsible for oxidizing perillyl alcohol into perillic acid have been identified (*cymB* and *cymC* , encoding cumic alcohol and aldehyde dehydrogenases respectively). It would be useful to compare CymBC and CdGe/GaDH in a future study for improving geranate and/or perillic acid production. To our knowledge, there are no dehydrogenases that can convert nerol into nerolic acid with high product specificity. Therefore, Ge/GaDH should be useful in microbial production of nerolic acid from nerol or simpler building blocks.

We found that farnesol (C15) could only be partially oxidized into its aldehyde, farnesal, by **BGA01** . Farnesoic acid was not detected in the reaction mixture and a large amount of farnesol was not utilized. These data provide useful information on the substrate scope of CdGeDH and CdGaDH. Several plant and insect farnesol dehydrogenase and farnesal dehydrogenase have been characterized during the past decade (Bhandari et al., 2010; Mayoral et al., 2009; Satyaveanthan et al., 2021; Seman-Kamarulzaman et al., 2016; Zifruddin et al., 2021). These enzymes could be employed to produce farnesoic acid from farnesol or isopentenols following the workflow established in our study.

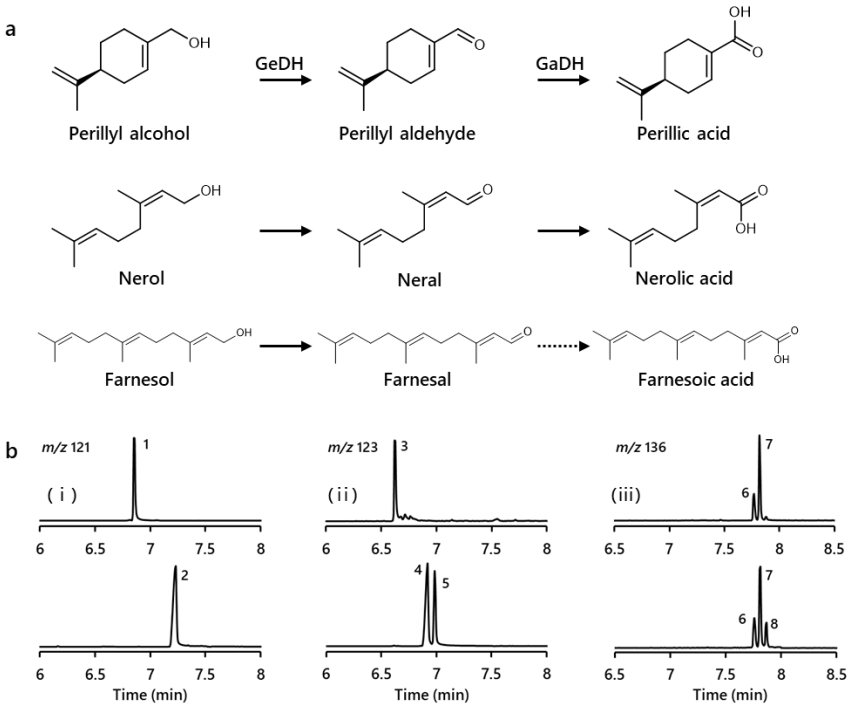

Figure 4 . **a)** Oxidation of perillyl alcohol, nerol and farnesol to aldehyde and carboxylate forms. **b)** GC/MS extracted ion chromatograms (EIC) of aqueous phase sample of biotransformation of (i) perillyl alcohol, (ii) nerol and (iii) farnesol with **BGA01**(lower row) and its parent strain (upper row). **BGA01** harbored the plasmid overexpressing CdGeDH and CdGaDH under the control of P_{T7}. Peaks numbered on each EIC are identified as 1, perillyl alcohol; 2, perillic acid; 3, nerol; 4, nerolic acid; 5, geranic acid; 6 & 7, farnesol; 8, farnesal.

CONCLUSION

In this study, we successfully constructed geranate-producing *E. coli* strains via IUP. We applied a two-stage fermentation protocol to increase the product titer. We found a competing pathway for geranate production, and optimized the expression level of the oxidoreductases to reduce by-product formation. The best strains produced 764 mg/L of geranate from 2 g/L isopentenols within 24 h, among the highest geranate titers reported in the literature. To our knowledge, this is the first study demonstrating the engineering of *E. coli* as microbial cell factory for *de novo* production of geranate using simple and cheap isopentenol substrates. The optimization approaches followed in this study may be applied to similarly reduce by-product formation in the production of other value-added isoprenoid compounds. We suggest that follow up studies improve the substrate specificity of CdGeDH and CdGaDH via protein engineering, and thus provide a basis host cell for further increasing the geranate titer through genetic and process engineering. A potential challenge might be the toxicity of geranate to *E. coli*at higher concentrations. This issue could be addressed by using a more robust host (e.g., *Pseudomonas* species), evolving the engineered*E. coli* in the presence of geranate, and/or sequestering geranate on charged resin or in a solvent.

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AUTHOR CONTRIBUTIONS

Qiuchi Pan and Kang Zhou conceived and designed the research. Xiaoqiang Ma contributed key materials and analysis tools. Qiuchi Pan, Xiaoqiang Ma, Yurou Liu, Hong Liang performed the analysis. Qiuchi Pan wrote the original draft of the manuscript. Qiuchi Pan, Xiaoqiang Ma, Kang Zhou and Gregory Stephanopoulos edited the manuscript.

CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

DATA AVAILABILITY STATEMENT

All of the data used to support the claims of this study have been presented in the form of figures and/or tables, which are available in this manuscript.

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